



**Embryonic stem cells in the pig
characterization and differentiation into neural cells**

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Embryonic stem cells in the pig: Characterization and differentiation into neural cells.

PhD thesis

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2010

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Cover illustrations: The cover shows a pig from which a blastocyst is isolated and cultured in-vitro to produce an outgrowth colony, which is visualized by staining with OCT4. Following differentiation into neural progenitor cells, visualized by staining with NESTIN and DAPI, the cells can be transplanted back into a porcine disease model, thereby constituting a large animal model of human stem cell therapy.

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Preface

This thesis is based on the Ph.D project carried out from 2007-2010 at Faculty of Life Science (LIFE), University of Copenhagen (KU), Denmark, where the experimental work was mainly performed. A part of the project was also performed in collaboration with Institute of Molecular Animal Breeding and Biotechnology, Ludwig Maximilian University (LMU), Munich, where I have spent eight months. The main supervisors are Professor Poul Hyttel and Postdoc Vanessa Hall at LIFE, KU with the co-supervisors Associate Professor Bolette Bjerregaard from LIFE, KU and Associate Professor Morten Meyer from University of Southern Denmark. Finally, Postdoc Nikolai Klymiuk was unofficial supervisor at LMU.

The work presented in this thesis includes an introduction including the objectives of the study, followed by a general background describing the relevant topics and techniques. Methods and materials as well as results are presented in three different manuscripts (see below), which are all related to the culture of porcine embryonic stem cells. Finally, in a discussion, conclusion and perspective, a parallel between the results and the literature is drawn. A review article on porcine embryonic stem cells is included at the end.

Publications originating from this study

- Manuscript I. Rasmussen MA, Wolf XA, Schauser K, Jensen AT, Schmidt M and Hyttel P. OCT4 expression in outgrowth colonies derived from porcine inner cell masses and epiblasts.
- Manuscript II. Rasmussen MA, Klymiuk N, Wüensch A, Kurome M, Kessler B, Petkov SG, Hall V, Vejlsted M, Høst T, Hyttel P, Wolf E. Analysis of porcine *OCT4* and *NANOG* promoter regions and expression in pluripotent cells and transgenic embryos.
- Manuscript III. Rasmussen MA, Hall VJ, Hyttel P. Directed differentiation of porcine epiblast-derived neural progenitor cells to mature neurons and glia
- Article I. Maddox-Hyttel P, Wolf XA, Rasmussen MA and Schauser K (2007). Embryonic stem cells in pig and cattle: Derivation, culture and potential applications. *Acta Scientiae Veterinariae* 35(Supl. 3):823-830

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1. Acknowledgement

First and foremost I would like to thank my supervisor Professor Poul Hyttel for giving me the opportunity to carry out this PhD and for encouraging me to pursue my goals. It has been a pleasure working under his inspiring supervision and I hope to continue the good collaboration and friendship in the future. I also owe great thanks to Postdoc Vanessa Hall for supporting me in developing my skills in the laboratory as well as for detailed corrections and feedback of scientific work. Furthermore, I would like to thank Postdoc Nikolai Klymiuk from LMU, who has taught me molecular cloning techniques and shown great hospitality during my stay in Munich. I would also like to thank my former supervisor, Kirsten Schauser as well as my co-supervisors Associate professors Morten Meyer and Bolette Bjerregaard.

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Finally, I would like to thank my family and friends for always supporting me and keeping up my spirit, particularly my girlfriend Katrine for her love, support and encouragement through the process.

2. List of abbreviations

AA	Ascorbic acid	NESTIN	Neuroepithelial stem cell protein
AD	Alzheimer's Disease	NCAM	Neural cell adhesion molecule
ALS	Amyotrophic lateral sclerosis	NCSU23	North Carolina State University 23
BAC	Bacterial artificial chromosome	NF	Neurofilament protein
bFGF	Basic fibroblast growth factor	NPC	Neural progenitor cell
BDNF	Brain-derived neurotrophic factor	NURR1	Nuclear receptor related 1
BMP	Bone morphogenetic protein	O4	Oligodendrocyte marker 4
BSA	Bovine serum albumin	OB	Olfactory bulb
CDX2	Caudal-related homeobox 2	OC	Outgrowth colony
CMV	Cytomegalovirus	OCT4	Octamer binding protein 4 (also known as POU5F1)
dEGFP	destabilized enhanced green fluorescent protein	P75	p75 Neurotrophin R
dERFP	destabilized enhanced red fluorescent protein	PAX6	Paired box 6
DMEM	Dulbecco's modified eagles medium	PBS	Phosphate buffered saline
DNA	Deoxyribonucleic acid	PD	Parkinson's Disease
EGC	Embryonic germ cell	PDGF	Platelet derived growth factor
EGF	Epidermal growth factor	pESC	Porcine embryonic stem cell
EGFP	Enhanced green fluorescent protein	PFA	Paraformaldehyde
EpiSC	Epiblast stem cell	PFF	Porcine fetal fibroblast
ERFP	Enhanced red fluorescent protein	PGC	Primordial germ cell
ERK	Extracellular regulated kinase	PGK1	Phosphoglycerate kinase 1
ESC	Embryonic stem cell	piPSC	Porcine induced pluripotent stem cell
FBS	Fetal bovine serum	pNPC	porcine neural progenitor cell
FGF	Fibroblast growth factor	POU5F1	POU domain, class 5, transcription factor 1 (also known as OCT4)
FGFR	FGF receptor	PVA	Polyvinyl alcohol
FOXD3	Forkhead Box D3	RA	Retinoic acid
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	REX1	Reduced expression protein 1
GDNF	Glial cell line-derived neurotrophic factor	RFP	Red fluorescent protein
GFAP	Glial fibrillary acidic protein	RNA	Ribonucleic acid
GFP	Green fluorescent protein	RT-PCR	Reverse transcription polymerase chain reaction
GP130	Glycoprotein 130	SCID	Severe combined immunodeficiency
GSK3	Glycogen synthase kinase 3	SCNT	Somatic cell nuclear transfer
HD	Huntington's Disease	SGZ	Subgranular zone
hESC	Human embryonic stem cell	SHH	Sonic hedgehog
hiPSC	Human induced pluripotent stem cell	SMAD	Similar to mothers against decapentaplegic
hNPC	Human neural progenitor cell	SMGT	Sperm mediated gene transfer
ICM	Inner cell mass	SOX2	SRY-related HMG-box protein 2
IRES	Internal ribosome entry site	SSEA	Stage-specific embryonic antigen
iPSC	Induced pluripotent stem cell	STAT	Signal transducer and activation of transcription
IVF	In vitro fertilized	STO	SIM mouse embryo-derived thioguanine and ouabain resistant
IVP	In vitro produced	SVZ	Subventricular zone
JAK	Janus-associated tyrosine kinase	TBP1	Tata-box binding protein
KSR	Knockout serum replacement	TGFβ	Transforming growth factor beta
KLF4	Kruppel-like factor 4	TH	Tyrosine hydroxylase
LIF	Leukemia inhibitory factor	TRA	Tumour rejection antigen
MCS	Multiple cloning site	TUJ1	β-tubulin III
MBP	Myelin basic protein	VZ	Ventricular zone
MEK	Mitogen-activated protein kinase	Wnt	wingless
mESC	Murine embryonic stem cell		
MEF	Mouse embryonic fibroblast		
miPSC	Mouse induced pluripotent stem cell		
mNPC	Mouse neural progenitor cell		
mRNA	messenger RNA		
MS	Multiple sclerosis		

3. Summary

Recent advances in human embryonic stem cell (ESC) research brings hope for a potential cure of severe diseases which are incurable with today's medical treatments. The pig is frequently used as a medical model for surgery and organ transplantation due to its similar anatomy and physiology to human. Yet, in spite of the obvious advantage of this species as a model organism of human stem cell therapy, little is known about porcine ESCs and their differentiation potential. In this PhD study, a method for derivation and evaluation of porcine ESC-like cultures was established. Outgrowth colonies (OCs) from the inner cell mass (ICM) or epiblast presented nuclear localized OCT4 staining, which correlated with an ESC-like morphology and reverse transcriptase PCR with *OCT4*, *NANOG* and *SOX2* showed that these markers were expressed as well. However, when subjected to passage, the ESC-like cells quickly lost expression of *OCT4* and other pluripotency markers. In contrast to previous reports, OCT4 was found to be localized exclusively in the epiblast of hatched in-vivo blastocysts, which underlines its importance as a pluripotency marker in the pig. Furthermore, promoter regions of *OCT4* and *NANOG* were isolated and aligned with other mammals and putative transcription factor binding sites were identified, including *OCT4*, *SOX2*, *KLF4* and *SMADs*, which showed highest sequence identity with corresponding binding sites in other ungulates and primates. Furthermore, fluorescent reporter constructs of *OCT4* and *NANOG* were generated and analysed in pluripotent cells and porcine pre-implantation embryos by use of sperm mediated gene transfer (SMGT) and somatic cell nuclear transfer. Both reporter constructs showed fluorescence in 2-cell to early morula stage embryos and fluorescence of the *NANOG* reporter was localized exclusively in the ICM of SMGT produced blastocysts. Directed differentiation of epiblast cells into neural cells was accomplished using a coculture protocol and a neural progenitor cell (NPC) line was isolated and maintained in culture for more than two months without losing expression of common NPC markers. When subjected to differentiation, mature neurons as well as astrocytes and oligodendrocytes were generated. Overall, these findings have brought the pig one step closer as a model of human stem cell therapy.

4. Sammendrag (Danish summary)

Fremskridt i menneskelig embryonal stamcelleforskning har bragt håb om en mulig helbredelse af sygdomme, som er uhelbredelige med nutidens medicinske behandlinger. Grisen bliver ofte brugt som medicinsk model for kirurgi og organtransplantation på grund af dens lignende anatomi og fysiologi til mennesket. Men på trods af grisens åbenlyse fordel som modelorganisme for menneskelig stamcelleterapi, har man kun en begrænset viden om embryonale stamceller fra svin samt deres differentierings potentiale. I dette PhD studie blev en metode til derivering og evaluering af ES-lignende kolonier etableret. ES-lignende kolonier, etableret fra den indre cellemasse eller epiblasten, viste kernelokaliseret OCT4 farvning, som korrelerede med en embryonal stamcellelignende morfologi og revers-transkriptase PCR med *OCT4*, *NANOG* og *SOX2* viste, at disse markører også var udtrykt i kolonierne. Cellerne mistede dog hurtigt udtrykket af OCT4 samt andre pluripotensmarkører når de blev udsat for passage. I modsætning til tidligere observationer, blev OCT4 udelukkende lokaliseret i epiblasten af hatched in-vivo blastocyster, hvilket understreger dens betydning som pluripotensmarkør hos svin. Promotorregioner fra *OCT4* og *NANOG* blev isoleret og sammenlignet med andre pattedyr og potentielle transskriptionsfaktor bindingssteder blev identificeret, såsom *OCT4*, *SOX2*, *KLF4* og *SMAD*, som mest lignede tilsvarende bindingssteder i andre store husdyr og primater. Fluorescerende rapporterkonstrukter for *OCT4* og *NANOG* blev endvidere genereret og analyseret i præ-implanterede embryoner ved hjælp af sædmedieret genoverførsel (SMGT) og somatisk kernecelletransplantation. Begge rapporterkonstrukter viste fluorescens fra 2-celle til tidlig morula-stadie embryoner med *NANOG* rapporterkonstruktet lokaliseret udelukkende i den indre cellemasse af SMGT producerede blastocyster. Differentiering af epiblast celler til neurale celler blev opnået ved dyrkning i co-kultur og en neural stamcellelinje blev isoleret og dyrket in-vitro i mere end to måneder uden at miste udtrykket af neurale stamcellemarkører. Når cellerne blev differentieret blev der endvidere dannet neuroner samt astrocyter og oligodendrocyter. Tilsammen har disse resultater bragt grisen et skridt nærmere som modelorganisme for menneskelig stamcelleterapi.

5. Introduction and objectives

The isolation of embryonic stem cells (ESCs) from the mouse almost 30 years ago is considered a hallmark of modern biotechnology and the scientific as well as the public interest in these cells has increased ever since. The reason for this is apparent: ESCs possess a unique ability to differentiate into all the 230 specialized cell types of the body in addition to a capacity of indefinite self-renewal. These fascinating characteristics render ESCs highly interesting as a means to study cellular development in-vitro and as a biomedical candidate to replace senescent and diseased cells of the body.

12 years ago, ESCs from human blastocysts were furthermore derived, bringing hope for thousands of people suffering from incurable diseases such as Parkinson's and Alzheimer's Disease. However, this brought along an ethical aspect as well, as destruction of human blastocysts was considered unethical by many people. Human ESCs possess the same characteristics as their murine counterparts, however, the germline potential, which is considered the ultimate proof of pluripotency has, due to ethical reasons, never been shown. In the recent years, there has been a paradigm shift in the public opinion, as more and more people are realizing the therapeutic potential of hESC and the necessity to promote research within this field. Furthermore, the recent discovery of induced pluripotent stem cells could obviate the need for embryo derived stem cells in the future.

With the stage set for human cell replacement therapy, the need for an animal model of stem cell therapy, which is phylogenetically close to humans, has never been greater. The pig has a comparable anatomy and physiology and similar size and complexity of organs to humans and with the recent sequencing of the genome as well as improvements in transgenic methods such as somatic cell nuclear transfer (SCNT), this species could constitute an important large animal model of human stem cell therapy. However, a major impediment is the lack of stable ESC lines in this species. Although several attempts have been made over the last 20 years no porcine ESC lines have so far proved capable of maintaining pluripotency in long-term cultures.

5.1. Main objectives of the PhD study:

The main objectives of the PhD study was:

- To derive ESC-like cultures from porcine pre-implantation embryos and characterize these with respect to pluripotency marker expression.
- To establish porcine reporter constructs specific for *OCT4* and *NANOG* and test these in pluripotent cells and transgenic embryos.
- To evaluate the potential of in-vitro cultured epiblast cells to differentiate into neural stem cells and mature neurons.

6. Background

6.1. *Embryonic development of the pig*

Embryonic development, which is defined as the time between fertilization and birth, is divided into different phases including cleavage, gastrulation, organogenesis and gametogenesis (Gilbert SF 1949). It is evident that the pig is fundamentally different from both humans and mice with respect to significant aspects of their embryonic development (Oestrup *et al.* 2009). A thorough understanding of the embryonic phases from fertilization to neurulation is important for identifying the optimal stage for derivation of ESCs and for identification of factors involved in neural differentiation in the pig.

6.1.1. Fertilization

Conception is the foundation of all mammalian organisms and can be defined as the formation of a new organism by fusion of the male and female gamete (Patten BM 1948). Fertilization occurs in the ampullary region of the oviduct and the embryos reach the uterus about 2 days after ovulation (Szollosi & Hunter 1973; Norberg 1973b; Hyttel *et al.* 1989). In the pig and cow, loss of cumulus investment allow the spermatozoon to contact the zona pellucida (ZP), after which the acrosome reaction occurs. Upon penetration of the ZP, syngami, which is defined as fusion of the two gametes, occurs. The result is formation of the zygote, i.e. the one-cell fertilized egg, which possesses the unique embryonic genome. Upon gamete fusion, the content of the spermatozoon including the nucleus, is expelled into the ooplasm along with the sperm plasma membrane being incorporated into the mosaic plasma membrane of the zygote (Laurincik *et al.* 1995). Along with this process, the content of the cortical granules are released into the perivitelline space by exocytosis, establishing a block against polyspermic fertilization. Furthermore, meiosis is resumed and maternal chromatin is advancing through ana- and telophase II. The maternal and paternal chromatin are surrounded by nuclear envelopes establishing two small pronuclei, which upon swelling to their large spherical shape, migrate to a close apposition slightly off centre in the zygote. Breakdown of the envelopes which is observed at about 24 h after ovulation results in synkaryosis, although an actual fusion of pronuclei does not occur in the pig (Hyttel *et al.* 1988). The zygote is said to be totipotent, which means “entire power” and is defined as the ability to give rise to all the cells of the body (Sheridan & Harris 2009). Totipotency is maintained until the 4-cell stage.

6.1.2. Cleavage

After fertilization, a series of mitotic cell divisions called cleavages occur, which results in progressively smaller cells without any change in the overall embryo size (Norberg 1973a). During the next 2-3 days this progressive increase in cell number, now called blastomeres, generates first a 2-cell, then a 4-cell and finally an 8-cell embryo (Figure 1). In the pig, the major genome activation occurs during the third cell cycle in the 4-cell embryo (Tomanek *et al.* 1989; Hyttel *et al.* 2000), in which formation of a fibrillogranular nucleolus signals the activation of the ribosomal RNA, required for protein synthesis and viability (Hyttel *et al.* 2000). In contrast, in the mouse and human, the major genome activation occurs after the second and fourth cell cycle, respectively (Ostrup *et al.* 2009). At the 8-16 cell stage, formation of tight junctions and desmosomes between the blastomeres initiates a process known as compaction in which the blastomeres begin to cluster together in a tight group, defined as a morula (Norberg 1973a).

6.1.3. Blastulation

The subsequent stage, which includes the first polarization of the embryo, is known as blastulation. Following compaction, an outer cell layer, called the trophectoderm develops, which actively transports fluid into the embryo, thereby creating a blastocyst cavity. In addition, a group of inner blastomeres gather at one pole to form the inner cell mass (ICM). The ICM cells are said to be pluripotent, which means “many potent” and can be defined as the ability to form all the cells of the body, but not the extra-embryonic tissues (Sheridan & Harris 2009). At this stage, the embryo is defined as a blastocyst, which is apparent from approximately Day 5 in the pig (Figure 1) (Hyttel & Niemann 1990) and at Day 3.5 and 5 in the mouse and human, respectively (Hall 2008). In the mouse, the ICM cells are most often used for derivation of ESCs (Ginis *et al.* 2004). Ultrastructural analysis of porcine Day 5 embryos showed that the cells of the ICM contain 1-2 nucleoli per nucleus, and are poor in cytoplasmic organelles (Norberg 1973a).

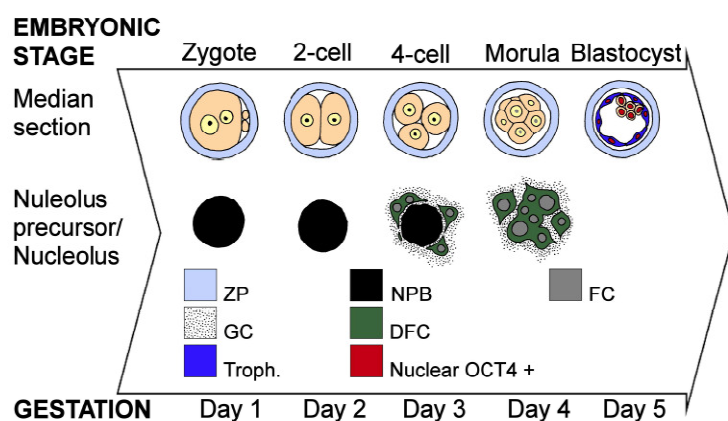


Figure 1. Schematic illustration of the development of the pre-hatching porcine embryo through embryonic genome activation and initial blastulation. Note that nucleolar precursor bodies (NPB) develop into nucleoli with fibrillar centres (FC), dense fibrillar component (DFC) and granular component at the four-cell stage, and that nuclear OCT4 expression is seen in both trophectoderm and inner cell mass (ICM) of the day 5 blastocyst.

6.1.4. Hatching

Hatching of the embryo, occurs after blastulation. At this point, major differences between porcine, murine and human embryonic development becomes apparent. The porcine blastocyst develops inside the ZP until approximately Day 7, at which stage the pressure on the ZP becomes too strong, resulting in rupture and hatching from it (Hyttel P *et al.* 2010). Around the same time, the ICM is in the process of separating into two distinct cell populations (Oestrup *et al.* 2009). The most ventral part flattens to form the hypoblast, which will finally line the inside of the embryo, whereas, the remaining ICM cells form the epiblast (Figure 2). This differs in mouse and human embryos, where the epiblast is formed prior to hatching around day 3.5 – 4.5 and day 6, respectively (Hall 2008). In the pig, the epiblast is defined as a proliferative epithelium containing tight junctions and desmosomes, which form a seal with the trophectoderm to separate the internal embryonic environment from the external uterine environment (Flechon *et al.* 2004). In humans, the epiblast stage is most often used for derivation of ESCs (Chen *et al.* 2009). Around day 9 of gestation, another major difference between porcine, murine and human embryonic development becomes apparent. In the pig, the epiblast becomes exposed to the uterine environment, which is caused by degradation of the polar trophectoderm called the Rauber's layer, after which it is referred to as the embryonic disc (Oestrup *et al.* 2009). The embryo is then defined as being in the pre-streak stage (Vejlsted *et al.* 2006a). The amniotic cavity is formed later by fusion of the extra-embryonic tissues. In contrast, in mice and humans, the embryonic disc is formed inside the embryo by a process which involves cavitation of the epiblast in which the amniotic cavity is formed and the innermost cells sealing this cavity make up the embryonic disc (Oestrup *et al.* 2009). Around Day 11-12, the porcine embryonic disc develops into an oval shape containing a crescent-shaped thickening in the posterior end due to accumulation of cells (Vejlsted *et al.* 2006a). This marks the pre-streak stage II and is the first sign of anterior – posterior polarity of the embryo proper (Maddox-Hyttel *et al.* 2003). Pluripotency is maintained in the epiblast until gastrulation occurs.

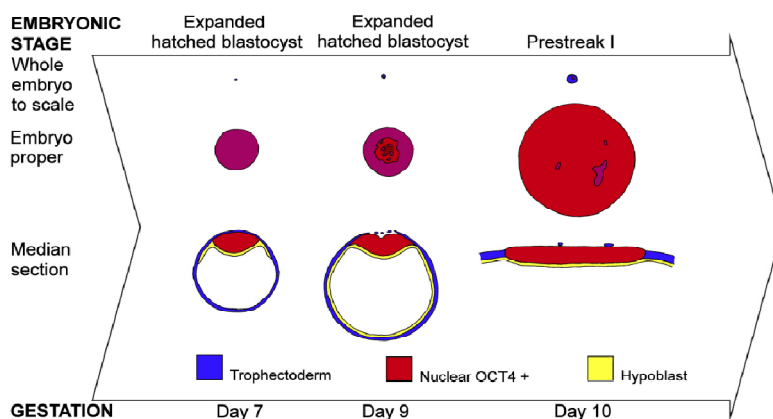


Figure 2. Schematic illustration of the post-hatching porcine embryo through late blastulation. Note that the hypoblast develops inside the embryo, that nuclear OCT4 expression becomes restricted to the epiblast, and that the Rauber' layer is lost. (Modified from Oestrup *et al.* 2009).

6.1.5. Gastrulation

At Day 12-13, the crescent-shaped thickening gathers at the posterior end of the disc and from this structure, the primitive streak extends anteriorly in the midline of the disc, marking the onset of gastrulation (Figure 3) (Vejlsted *et al.* 2006a). In the mouse it has been shown that members of the transforming growth factor β (*Tgfb*) family such as *Nodal* have a profound role in initiating and maintaining primitive streak formation (Brennan *et al.* 2001), whereas bone morphogenic protein 4 (*Bmp4*) was found to be required for the generation of primordial germ cells (PGCs) (Lawson *et al.* 1999). The primitive streak elongates in an anterior direction to around two thirds of the embryo, forming a primitive groove in the midline, through which cell movement from the posterior part of the epiblast occurs (Vejlsted *et al.* 2006b). The result is an epithelial-mesenchymal transition of epiblast cells to either meso- or endodermal origin in response to different concentrations of signaling factors, depending on the spatial movement through the primitive streak (Oestrup *et al.* 2009). In the anterior part of the streak, a region called the Spemann's organizer is formed by synergistic input from *Activin* and *Wnt* signaling pathways (Watabe *et al.* 1995). The structure was named after the german embryologist Hans Spemann who showed that transplantation from one *Xenopus* embryo to another induced a secondary embryonic primordia (Spemann H & Mangold H 1924). When the primitive streak starts to regress, the primitive node gives rise to a mesodermal structure called the notochord, which plays important parts in the subsequent neural induction and patterning. Concurrently, the trophoblast begins to elongate dramatically from a 1 cm ovoid structure to around a 1 m thin filamentous structure (Figure 3). This striking feature may be the cause of a prolonged pre-implantation period which is followed by non-invasive placentation in the pig compared to the mouse and human implantation (Flechon *et al.* 2004).

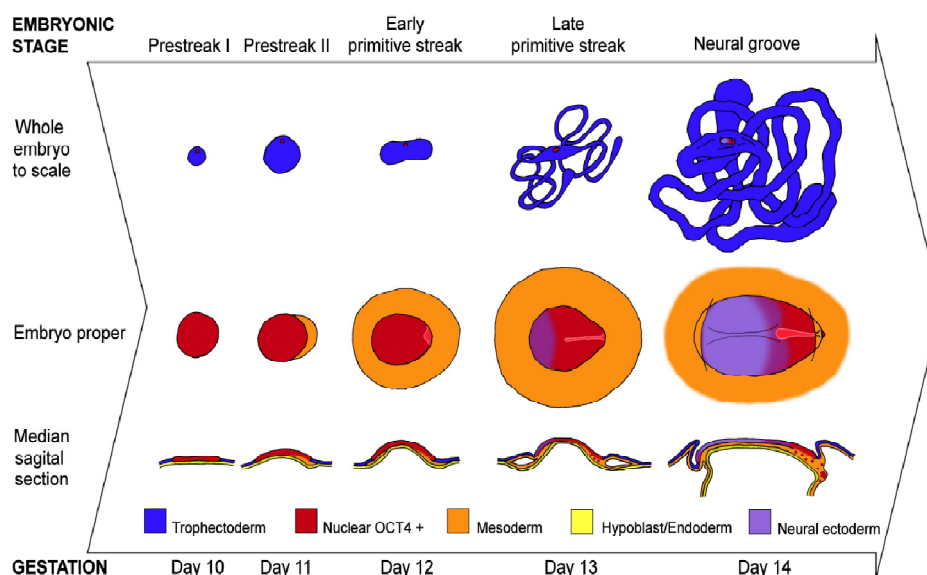


Figure 3. Schematic illustration of gastrulation in the porcine embryo. Note that the embryo elongates, that a posterior crescent including mesoderm ingression forms, that the nuclear OCT4 expression becomes posteriorly restricted, that OCT4 positive primordial germ cells allocate to the dorsal hindgut and a posterior cluster, and that amniotic folds form anteriorly and posteriorly. (Modified from Oestrup *et al.* 2009).

6.1.6. Neurulation

The notochord has been shown to play a crucial role in neural induction, which is defined as the step when epiblast cells become ‘specified’ as neural cells (Wilson & Hemmati-Brivanlou 1995; Wilson & Edlund 2001). Studies on neural induction in *Xenopus* have shown that secretion of noggin (Smith & Harland 1992), chordin (Sasai *et al.* 1995) and follistatin (Hemmati-Brivanlou *et al.* 1994) from the notochord inhibit the action of members of the *tgfb* pathway such as bmp’s, which would otherwise instruct the ectoderm into an epidermal fate (Colas & Schoenwolf 2001). In the chicken it was furthermore shown that cells receiving fibroblast growth factor (FGF) (Wilson *et al.* 2000), in addition to the inhibitory signals, will form a neural plate in the anterior part of the epiblast with the formation progressing in a anterior-posterior direction. The neural plate is a keyhole-shaped structure with a broad anterior and a narrow posterior region (Hyttel P *et al.* 2010). During the third week of development, two lateral folds start to form on each side of the midline, thereby creating a neural groove. At the 5-7 somite developmental stage, the neural folds fuse to form a neural tube (Van Straaten *et al.* 2000) which is the site of initial specification of neurons as well as the origin of the major components of the nervous system (Colas & Schoenwolf 2001). The closure of the neural tube occurs anteriorly and posteriorly in a zipper-like fashion (Sadler 2005) and is mediated by glycoproteins which hold the folds in place until more permanent cell to cell contacts can be established (Sadler 2005). In contrast to the mouse, which have two anterior neuropore closing sites, the pig has only a single, which closes at the 22th somite stage, whereas, the posterior neuropore closes at the 28th somite stage (Van Straaten *et al.* 2000). The notochord, furthermore plays an important part in the patterning of neurons in the neural tube (Vejlsted *et al.* 2006a). Neural patterning occurs in response to a gradient of sonic hedgehog (SHH) secreted from the notochord and BMPs and wingless (WNT) secreted from the neuroectoderm, which have opponent and antagonistic functions along the dorso-ventral axis of the neural tube (Liem, Jr. *et al.* 2000). The formation of the neural tube, which will later give rise to the central nervous system, is known as primary neurulation. The embryonic development of the pig ends at Day 35, with the formation of the major organs, after which the fetal stage begins (Hyttel P *et al.* 2010).

6.2. Embryonic stem cells

6.2.1. Pluripotency and self-renewal

In general, three essential characteristics define ESCs (Thomson & Marshall 1998): Firstly, ESCs are derived from the pre-implantation or peri-implantation embryos, more precisely from the ICM or the epiblast. Secondly, ESCs are capable of prolonged undifferentiated proliferation, which is defined as self-renewal, and finally, ESCs are able to form derivatives of the three embryonic germ layers, ectoderm, mesoderm and endoderm as well as the germ line. These characteristics, which are particularly unique to ESCs, makes them highly attractive for studying developmental processes in-vitro, as well as an interesting cell type for use in regenerative medicine (Thomson *et al.* 1998).

6.2.1.1. Derivation of mouse ESC

ESCs were first derived in the mouse (mESCs) in 1981 by two separate research groups (Evans & Kaufman 1981; Martin 1981). Morphologically, they are described as small, round cells with a large nucleus surrounded by a narrow band of non-granular cytoplasm (Robertson EJ 1987). Ultra-structural analysis showed that mESCs contain some tight- and gap-junctions, especially close to the surface of colonies (Ginis *et al.* 2004). mESCs typically grow in tight, rounded, multilayered colonies on feeder cells such as inactivated mouse embryonic fibroblasts (MEF) (Robertson EJ 1987). However, mESCs can also be cultured in feeder free conditions in the presence of leukemia inhibitory factor (LIF) and serum (Smith 2001), or in more defined conditions with LIF and bone morphogenic protein 4 (BMP4) (Ying *et al.* 2003). When mESCs are left without passage for approximately a week, they begin to differentiate in the periphery of the colony (Robertson EJ 1987).

mESCs are typically derived from a few inbred strains of mice, such as 129 and C57BL/6 (Nagy & Vintersten 2006). However, mESCs have also been derived from non-permissive strains by means of transgenic methods such as selective ablation (McWhir *et al.* 1996; Gallagher *et al.* 2003) and by overexpression of kruppel-like finger 4 (KLF4), C-MYC or small molecules (Hanna *et al.* 2009). When cultured in suspension, mESC have been shown to form embryoid bodies (EBs) consisting of tissue from the three germ lineages, ectoderm, mesoderm and endoderm (Martin 1981). Moreover, when mESCs are injected into nude mice in-vivo, they form teratomas, which is a type of tumor consisting of tissues from the three germ lineages (Evans & Kaufman 1981). Mouse ESCs also possess the capacity to generate chimeric animals when injected into early embryos in-vivo and it is possible to breed these chimeric mice and produce offspring derived from the mESCs (Bradley *et*

al. 1984). In addition, germ line transmission was demonstrated by Nagy and co-workers, who showed that completely mESC derived chimeric mice could be obtained by aggregation with tetraploid embryos (Nagy *et al.* 1993). As tetraploid embryos were developmentally compromised at this stage and only formed placentas, mESCs cultured up to passage 14 was forced to form the entire embryo proper (Nagy & Vintersten 2006). In contrast, mESCs from non-permissive strains typically lack the ability to contribute to chimeras (Chen *et al.* 2005). Studies have shown that in-vitro cultured mESCs are capable of more than 250 population doublings without signs of reduced growth rate or abnormal karyotypes, while maintaining their pluripotency (Suda *et al.* 1987).

6.2.1.2. Derivation of human ESC

ESCs were derived from humans (hESCs) in 1998 by Thomson and colleagues (Thomson *et al.* 1998). The morphology of hESCs was described as being similar to mESCs, and ultrastructural analysis also showed formation of tight- and gap junctions between the cells, particularly at the edge of the colony (Park *et al.* 2004). However, hESCs tend to grow in more flat, loose colonies with a clear colony border when cultured on MEF feeder cells in medium containing basic fibroblast growth factor (bFGF) (Thomson *et al.* 1998). hESCs can also be maintained on STO feeder cells, which is a SIM mouse embryo-derived Thioguanine and Ouabain resistant cell line with the capability of dividing indefinitely (Park *et al.* 2003), on extracellular matrix (Klimanskaya *et al.* 2005) or even in chemically defined conditions (Yao *et al.* 2006). However, exogenous supplementation with ACTIVIN was required to maintain hESCs in the undifferentiated state, except when cultured on MEF feeder cells, which are known to produce this growth factor (Beattie *et al.* 2005). When hESCs are left without passage for one to two weeks, they start to differentiate from the inside of the colony in contrast to mESCs (Thomson & Marshall 1998).

Human ESC lines are most often derived from blastocysts which are obtained from excess in-vitro fertilized eggs, and thus, the quality of such embryos varies considerably (Rolletschek & Wobus 2009). Furthermore, derivation of hESCs from morula stage embryos (Strelchenko *et al.* 2004) and single blastomeres (Klimanskaya *et al.* 2006) have been reported. Recently, Chen and colleagues studied the optimal timing of hESC-derivation using laser assisted isolation of ICM from 584 frozen human embryos (Chen *et al.* 2009). hESC lines could be derived from embryos at days 5–9 after fertilization, with isolation on day 6 resulting in the most efficient derivation, corresponding to the time of hatching (Hall 2008). This was found to be consistent with a restricted localization of the pluripotency marker octamer binding protein 4 (OCT4; also known as POU5F1) to the early

epiblast and the trophectodermal marker, caudal-related homeobox 2 (CDX2), to the trophectoderm. Furthermore, hESC conditioned medium was reported to improve the derivation of hESC lines from low quality embryos (Chen *et al.* 2009).

The pluripotency of hESCs has been studied in-vitro by EB formation and in-vivo by teratoma formation in severe combined immunodeficient (SCID) mice (Thomson *et al.* 1998). In both cases, cells from the three germ layers were identified. In contrast, chimera contribution and germ line potential of hESCs has, due to ethical reasons, not been tested. Human ESCs can be maintained for several years in culture and undergo hundreds of population doublings (Hoffman & Carpenter 2005).

6.2.1.3. Attempts of porcine ESC derivation

Since the initial attempts of porcine ESC (pESC) derivation (Piedrahita *et al.* 1990; Notarianni *et al.* 1990), a range of attempts have been made to establish porcine ESCs (pESC) (for reviews see (Vackova *et al.* 2007; Keefer *et al.* 2007; Brevini *et al.* 2007a; Hall 2008; Talbot & Blomberg 2008; Blomberg *et al.* 2008)). However, none of the attempts have resulted in the derivation of stem cell lines which display the same pluripotency characteristics as human and mouse ESCs (Telugu *et al.* 2009). Instead, many cell lines appear to have some stem cell characteristics, and are thus termed stem cell-like.

In general, the morphology of pESC-like cells were reported to be similar to mESCs and hESCs with a high nuclear-cytoplasmic ratio, containing one or two nucleoli (Piedrahita *et al.* 1990). Ultra-structural analysis showed that isolated pig epiblasts from Day 8 embryos, cultured for 36 hours on STO feeder layers formed a monolayer of cells connected by tight junctions and desmosomes (Talbot & Garrett 2001). In addition, structures such as Golgi, mitochondria and microfilaments were more developed compared to uncultured epiblast cells. Interestingly, a solitary cilium was observed projecting from the apical surface of an epiblast cell (Talbot & Garrett 2001). In hESC, primary cilia has recently been shown to be involved in SHH signaling and is shown to play a critical role in hESC differentiation parallel to that in early embryogenesis. (Kiprilov *et al.* 2008).

At least 12 different groups have reported that pESC-like cells can generate cells representative of the three germ layers, when they are cultured as EBs (Vackova *et al.* 2007). However, the passage numbers, from which these cells are used, are not stated in these studies. In-vivo transplantation of

whole porcine blastocysts in mice gave rise to teratomas, but only in older stage embryos around Day 11-12 (Anderson *et al.* 1994). In accordance, pESC-like lines derived from Day 10-11 epiblasts also developed teratomas, whereas, cell lines from Day 5-6 blastocysts failed to do so (Piedrahita *et al.* 1990; Hochereau-De Reviers & Perreau 1993). It is possible that the late stages were already undergoing differentiation, as gastrulation initiates around Day 12 in the pig. In contrast, fetal (Notarianni *et al.* 1997) and live born chimeras (Anderson *et al.* 1994; Onishi *et al.* 1994; Nagashima *et al.* 2004) with germ line potential were produced by injection of freshly isolated ICM or early epiblast from Day 6-7 embryos into blastocysts, which substantiates that this stage is pluripotent. When subjected to in-vitro culture, ICM-derived cells cultured for less than 15 passages with passage every fourth days proved capable of integrating into chimeric embryos (Shiue *et al.* 2006). However, only a single report has described the production of a live born chimeric pig derived from pESC-like cells, but germ line potential was not reported (Chen *et al.* 1999). In our laboratory, we have obtained and cultured cells from the same pESC line, but were unable to detect essential markers of pluripotency such as *OCT4*, *NANOG* and *SOX2* (Personal communication, Petkov S and Hall V). The longest reported maintenance of a pESC-like cell line is around one year (Notarianni *et al.* 1990) or 90 passages (Talbot *et al.* 1993), however, these lines have not been characterized in-vivo.

6.2.2. Signaling pathways

6.2.2.1. Signaling pathways in mouse ESC

Pluripotency is maintained through different signaling pathways in the mouse and human, respectively (Valdimarsdottir & Mummery 2005). In mESCs, the janus-associated tyrosine kinase and signal transducer and activator of transcription 3 (JAK-STAT3), BMP4, WNT and mitogen-activated protein kinase and extracellular regulated kinases (MEK-ERK) are the major pathways maintaining self-renewal and pluripotency (Figure 4.) (Okita & Yamanaka 2006). LIF is known to support the undifferentiated state of mESCs by activating the transcription factor STAT3 through the JAK/STAT pathway (Smith *et al.* 1988) and BMP4 can enhance self-renewal and pluripotency of mESC by activating members of the inhibition of differentiation (*id*) gene family through the SMAD1/5/8 pathway (Ying *et al.* 2003). In addition, the WNT pathway has been shown to delay the onset of differentiation (Sato *et al.* 2004). Recently, a defined protocol in which a combination of three inhibitors targeting the FGF, MEK, and the glycogen synthase kinase 3 (GSK3) receptors resulted in efficient derivation and propagation of germline competent mESCs from a range of non-permissive mouse strains (Ying *et al.* 2008). Surprisingly, the same strategy proved to be successful in derivation of rat ESCs, which were capable of germ line transmission (Buehr *et al.* 2008). It was proposed that the key to ESC derivation could lie in the use of inhibitors to shield the native state from differentiation, rather than growth factor stimulation.

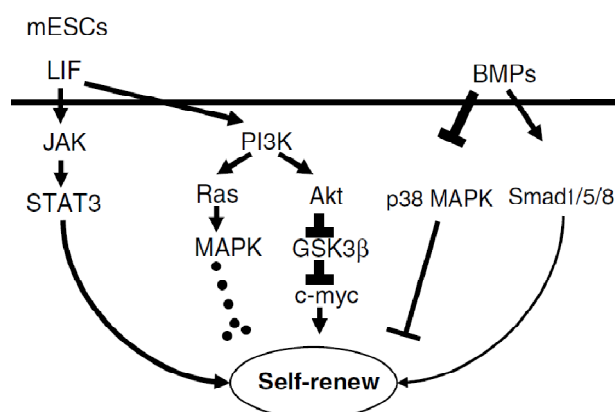


Figure 4. Key signaling pathway required for maintaining pluripotency of mouse embryonic stem cells. LIF signaling activates JAK–STAT3 to induce target genes essential for pluripotency, such as *c-myc*. *c-myc* is also regulated negatively by glycogen synthase kinase-3 (GSK3)β via inhibitory phosphorylation. Leukemia inhibitory factor also induces MAP kinase activation, which antagonizes self-renewal. Bone morphogenetic protein signals potentially function in two ways: (i) activation of Smad1/5/8-Id gene and (ii) suppression of p38 MAP kinase. (Modified after Ohtsuka *et al.* 2008).

6.2.2.2. Signaling pathways in human ESC

In contrast to mESCs, pluripotency in hESCs is maintained through the FGF, the ACTIVIN/NODAL and the MEK/ERK pathways (Figure 5) (Ohtsuka & Dalton 2008). bFGF signaling (Mummery *et al.* 1993; Levenstein *et al.* 2006) has been shown to occur through a paracrine network, in which bFGF binds to MEF cells and hESC derived fibroblast-like cells, which in turn produce factors such as insulin-like growth factor necessary for the survival of hESCs (Greber *et al.* 2007; Bendall *et al.* 2007). Furthermore, bFGF has been shown to repress BMP4 and its downstream effectors, SMAD1/5/8 (Xu *et al.* 2005). ACTIVIN/NODAL signaling through SMAD2/3 (Vallier *et al.* 2005; James *et al.* 2005), as well as the MEK/ERK pathway (Li *et al.* 2007) are both necessary to maintain the pluripotent state of hESCs. In addition, the WNT pathway has been shown to delay the onset of differentiation in hESCs (Sato *et al.* 2004).

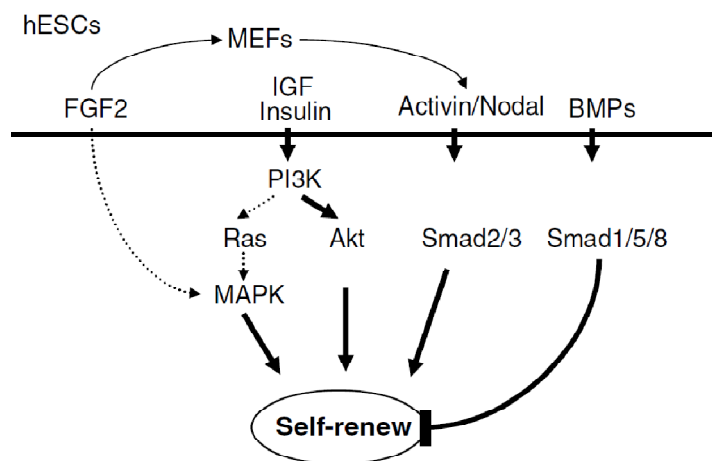


Figure 5. Key signaling pathway required for maintaining pluripotency of human embryonic stem cells. Fibroblast growth factor is an essential factor for hESCs self-renewal and functions in part by inducing Activin secretion from mouse embryonic fibroblasts. Activin/Nodal signaling is essential to support hESCs self-renewal via activation of Smad2/3. In contrast to mESCs, bone morphogenetic protein promotes hESCs differentiation toward trophoderm. (Modified after Ohtsuka *et al.* 2008).

6.2.2.3. Signaling pathways in porcine ESC

In contrast to the mouse and human, not much is known about the regulation of pluripotency in the pig. It has been reported that NOGGIN (an antagonist of BMP4 acting to inhibit differentiation in human ES cell) was expressed exclusively in the epiblast of Day 8 embryos (Blomberg *et al.* 2008). Furthermore, low levels of *LIF*, glycoprotein 130 (*GP130*), and *BMP4* were detected In Day 6 embryos, whereas bFGF and FGF receptor (FGFR) 1 and 2 were detected in Day 11 embryos (Hall *et al.* 2009). Since FGFR1 was located exclusively in the epiblast and bFGF was produced by the surrounding trophoderm it was speculated that, as in hESCs, a paracrine FGF signaling pathway could play a role in maintaining pluripotency at this developmental stage. It is, however, possible that other unknown pathways are involved in maintaining the pluripotent state in the pig.

6.2.3. Pluripotency markers

In ESCs, external signaling with growth factors leads to regulation of a number of genes ultimately resulting in the pluripotent state (Pan & Thomson 2007). These downstream targets have been the focus of much research and to date, a range of pluripotency markers have been identified in the mouse and human, of which some seem to be species-specific (Ginis *et al.* 2004). Three key transcription factors, OCT4, NANOG and SOX2, seem to be highly conserved and are commonly used to define pluripotent cells in rodents and primates (Thomson *et al.* 1998). In hESCs it has been reported that OCT4, NANOG and SOX2 bind simultaneously to more than 352 genes, thereby regulating their expression (Boyer *et al.* 2005). In addition, they bind to their own promoters, thereby forming an interconnected auto-regulation loop which maintain the ESC identity. In the pig, however, an atypical expression pattern of these pluripotency markers have been reported (Keefer *et al.* 2007; Hall *et al.* 2009), which casts doubt upon their role in maintaining the pluripotency network in this species. Hence, a comparison of the expression of core pluripotency markers in the pig and the mouse and human, is essential.

6.2.3.1. *Oct4*

Oct4 was first discovered in the mouse in 1990 (Okamoto *et al.* 1990; Scholer *et al.* 1990b) and was mapped to chromosome 17, close to the major histocompatibility complex (MHC) (Scholer *et al.* 1990a). OCT4 is considered the most valid marker of epigenetic reprogramming and pluripotency (Pesce & Scholer 2000; Pesce & Scholer 2001) and belongs to a group of proteins containing a POU-domain (Pit-Oct-Unc), which enables it to bind to the octamer consensus sequence ATGCAAAT. In conjunction with SOX2, OCT4 is known to upregulate the expression of *Nanog* (Rodda *et al.* 2005) and *Sox2* (Catena *et al.* 2004), in addition to its own expression (Figure 6) (Okumura-Nakanishi *et al.* 2005). Furthermore, it upregulates *Fgf4* which is essential to survival of post-implantation embryos (Yuan *et al.* 1995; Niwa *et al.* 2000). Multiple other downstream targets of OCT4 have been identified (Loh *et al.* 2006; Wang *et al.* 2006; Babaie *et al.* 2007; Kim *et al.* 2008a).

Oct4 is expressed in a tissue-specific manner in mouse pre-implantation embryos (Schoorlemmer *et al.* 1994). The expression starts from the 4-cell stage immediately after the genome activation and remains present in all cells until the morula stage (Ovitt & Scholer 1998). At the blastocyst stage, *Oct4* becomes restricted to the ICM (Dietrich & Hiiragi 2007). Following implantation, it is limited

to primitive ectodermal cells (Ovitt & Scholer 1998) and finally around gastrulation it becomes restricted to the PGC (Pesce & Scholer 2000). Studies have shown, that mice containing a homozygotic deletion of *Oct4* do not develop further than the blastocyst stage and do not contain an ICM but consists instead entirely of trophectoderm (Nichols *et al.* 1998). Hence, it was proposed that *Oct4* maintains totipotency (Yeom *et al.* 1996; Nordhoff *et al.* 2001). When Niwa and colleagues examined the regulation of *Oct4* in mESCs they found that it was regulated in a dose dependent manner in which downregulation of 150% induced trophectoderm differentiation, whereas, an upregulation of 50% induced hypoblast or mesoderm differentiation (Niwa *et al.* 2000). The authors proposed that *Oct4* functions as a “gatekeeper” of pluripotency, by preventing differentiation of cells into the trophoblast lineage, which is mediated through a downregulation of trophoblast-specific genes such as *Cdx2* (Figure 6) (Niwa *et al.* 2000).

Human *OCT4* was also identified close to the MHC on chromosome 6 (Takeda *et al.* 1992). In contrast to the mouse *Oct4*, at least two isoforms have been identified due to differential splicing, termed *OCT4A* and *OCT4B* (Cauffman *et al.* 2006). Whereas *OCT4A* seems to be specific for hESCs and hEGCs, *OCT4B* is also expressed in mature cells. However, the latter can be identified by its cytoplasmic localization (Atlasi *et al.* 2008). In addition, several *OCT4* pseudogenes have been identified, but these are not functionally active. The expression profile of *OCT4* in human pre-implantation embryos was first believed to correspond to the mouse, as a difference in *OCT4* expression was found between the ICM and the TE on the mRNA level (Hansis *et al.* 2000; Hansis *et al.* 2001). However, analysis of mRNA expression and protein staining later revealed *OCT4* expression in both of these cell types, from the morula to the blastocyst stage, indicating a different expression pattern than the mouse (Cauffman *et al.* 2005). Using immunocytochemistry, it was recently shown that OCT4 becomes confined to the ICM of the blastocyst from approximately Day 6, when the epiblast and hypoblast begin to form (Chen *et al.* 2009). In hESC, OCT4 is often used as a pluripotency marker, and is considered a master regulator of self-renewal (Atlasi *et al.* 2008).

In the pig, *OCT4* is located close to the MHC on chromosome 7 (Chardon *et al.* 2000). It does not seem to be specific for totipotent cells as immunocytochemical analysis revealed the presence of OCT4 protein in all the cells of the blastocyst, including the ICM and trophectoderm (Figure 1) (Kirchhof *et al.* 2000; Spencer *et al.* 2006; Keefer *et al.* 2007; Kuijk *et al.* 2008; Hall *et al.* 2009). After hatching, OCT4 was reported to be present in both the ICM and trophectoderm until Day 10-

13, after which it is downregulated (Kirchhof *et al.* 2000; Kuijk *et al.* 2008). However, other studies have shown that OCT4 becomes confined to the epiblast after hatching (Figure 2) (Flechon *et al.* 2004; Vejlsted *et al.* 2006a; Vejlsted *et al.* 2006b). Furthermore, Blomberg and colleagues found that *OCT4* displayed a restricted expression pattern to the epiblast of Day 8 blastocysts (Blomberg *et al.* 2008). An upregulation of *OCT4* after 24-48 hours of in-vitro culture was furthermore reported, which could preclude the onset of differentiation. At day 13, OCT4 is only found in PGCs, which allocate to the dorsal hindgut and a posterior cluster (Figure 3) (Vejlsted *et al.* 2006b). Studies on different types of porcine embryos have shown that nuclear transfer blastocysts had significantly lower expression of *OCT4* compared to in-vivo and in-vitro fertilized blastocysts (Kumar *et al.* 2007; Xing *et al.* 2009). The low level of *OCT4* expression in nuclear transfer blastocysts could reflect an incomplete reprogramming, or the presence of fewer cells within the embryo.

6.2.3.2. *Nanog*

Nanog was first identified in the mouse in 2003 (Wang *et al.* 2003; Chambers *et al.* 2003; Mitsui *et al.* 2003) and was named after a mythological Celtic land of eternal youth, Tir Nan Og. *Nanog* belongs to a family of proteins containing homeobox domains, and binds to the consensus sequence (C/G)(G/A)(C/G)C(G/C)ATTAN(G/C), where ATTA is a common homeobox DNA binding sequence (Mitsui *et al.* 2003). *Nanog* is believed to direct the ICM to an epiblast fate by downregulating of primitive endoderm specific genes such as *Gata6* (Figure 6) (Mitsui *et al.* 2003). Hence, *Nanog* acts one step later in development compared to *Oct4*.

Nanog is expressed in a tissue-specific manner in the mouse (Wang *et al.* 2003). The earliest expression has been detected in late morula and was confined to the ICM of blastocysts (Mitsui *et al.* 2003; Hart *et al.* 2004; Hatano *et al.* 2005). After implantation, *Nanog* is only expressed in the epiblast (Hatano *et al.* 2005). *Nanog* is downregulated during gastrulation when the epiblast cells enter the primitive streak, after which it is not detected again until formation of the genital ridge of the early gonads (Hart *et al.* 2004). Transcripts for *Nanog* are not detected in several adult tissues by Northern Blot, however, low levels of *Nanog* were reproducibly detected in many adult tissues by RT-PCR (Hart *et al.* 2004). Despite a delayed expression in pre-implantation embryos, the expression profile of *Nanog* seems to be quite similar to that of *Oct4*. When Mitsui and colleagues bred mice heterologous for *Nanog*, no homozygotic *-/- Nanog* mice were found, which was attributed to a lack of epiblast formation (Mitsui *et al.* 2003). However, it was later shown that mutant *Nanog -/-* mice were in fact able to survive to adulthood although contribution to the germ line was not ob-

served (Chambers *et al.* 2007). The authors concluded that *Nanog* is dispensible for expression of somatic pluripotency but is specifically required for formation of EGCs. Interestingly, overexpression of *Nanog* in mESCs has been shown to be sufficient to maintain pluripotency without the requirement of feeder cells or growth factors (Chambers *et al.* 2003). However, Hatano and colleagues noticed that mESCs positive for *Oct4*, were not always positive for *Nanog* (Hatano *et al.* 2005). This fluctuation was later confirmed by Chambers and colleagues, whom showed that *Nanog* is not strictly required for self-renewal of murine ESC but rather acts as a safeguard against differentiation (Chambers *et al.* 2007).

In human, NANOG protein was identified in the ICM of expanded blastocysts, but not in earlier developmental stages (Hyslop *et al.* 2005). This is in accordance with its function later in development compared to OCT4 (Mitsui *et al.* 2003). Like *OCT4*, two isoforms of *NANOG* have been detected in humans (Hart *et al.* 2004). Low levels of *NANOG* expression detected in many adult tissues by RT-PCR was attributed to the spliced variant. Furthermore, *NANOG* was found to be expressed in hESCs and embryonic carcinoma cells and downregulation with small interfering RNA resulted in upregulation of extraembryonic endoderm and trophoblast-specific genes, indicating that *NANOG* also function as a “gatekeeper” of pluripotency in humans (Hyslop *et al.* 2005).

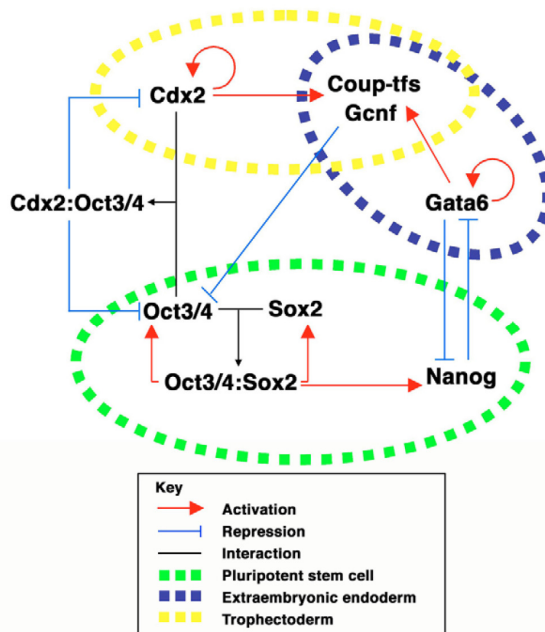


Figure 6. Transcription factor networks for pluripotent stem cells (green), trophoblast (yellow) and primitive (extraembryonic) endoderm (blue). Positive-feedback loops between *Oct4*, *Sox2* and *Nanog* maintain their expression to promote continuous ES cell self-renewal. *Cdx2* is autoregulated and forms a reciprocal inhibitory loop with *Oct4*, which acts to establish their mutually exclusive expression patterns. A similar regulatory loop exist for *Nanog* and *Gata6*. *Coup-tfs* and *Gcnf* act as a negative-feedback system to repress *Oct4* completely. (From Niwa *et al.* 2007).

In the pig, *NANOG* mRNA expression as well as protein staining was not observed in Day 6-8 blastocysts (Blomberg *et al.* 2008; Kuijk *et al.* 2008; Hall *et al.* 2009). Instead, *NANOG* was detected in several adult cells and tissues including porcine umbilical cord cells and porcine fetal fibroblasts (Carlin *et al.* 2006) as well as porcine brain, lung and liver (Blomberg *et al.* 2008). Together, these results points to a non-pluripotent role of *NANOG* in the pig. On the other hand, using immunocytochemistry, *NANOG* has been observed exclusively in the epiblast of Day 9 and 11 blastocysts (Hall *et al.* 2009) and studies have shown that *NANOG* transcripts were significantly upregulated in in-vivo blastocysts compared to in-vitro produced blastocysts (Kumar *et al.* 2007; Magnani & Cabot 2008; Xing *et al.* 2009).

6.2.3.3. Other pluripotency markers

Several other pluripotency markers have been described in the mouse and human, of which the most important are the transcription factors *Sox2* and *Rex1* as well as stage specific embryonic antigens (SSEA) -1, -2, -3 and -4 and the tumor rejection antigens (TRA) -1-60 and -1-81 (Ginis *et al.* 2004).

Sox2 is a member of the SRY-related HMG box gene family that encode transcription factors with a single DNA-binding domain. (Avilion *et al.* 2003). In the mouse, *Sox2* is expressed in the cells of the ICM and its descendant, the epiblast, whereas, in the trophectoderm it is localized in cytoplasm. *SOX2* has been found to regulate a range of genes associated with pluripotency, often in collaboration with *OCT4* (Catena *et al.* 2004). However, in contrast to *Oct4* and *Nanog*, *Sox2* is not an exclusive marker of pluripotency as it is also expressed in early neuroectodermal cells. In-vitro, *Sox2* is expressed in both mESCs and hESCs (Ginis *et al.* 2004). Studies of *SOX2* expression in the pig are limited, however, it was reported to be exclusively expressed in the epiblast of Day 9-11 embryos (Hall *et al.* 2009).

The zinc finger protein, *Rex1* is a transcription factor present in the mouse and human ICM and ESCs (Rogers *et al.* 1991). However, *Rex1* does not appear to be a specific marker in these species as it is also detected in the trophectoderm. In the pig, *REX1* seems to be specific for the epiblast of Day 8 blastocysts and could be a useful marker of pluripotency (Blomberg *et al.* 2008).

The cell surface markers are glycoproteins specifically expressed in early embryonic development (Mandal *et al.* 2006). Mouse ESCs express SSEA1 and -3, whereas, hESCs express SSEA3 and -4 (Ginis *et al.* 2004). In addition, SSEA-1 has been shown to be expressed in the Day 7 porcine epib-

last but not in the trophectoderm (Wianny *et al.* 1997) and in some cells of the Day 12 embryonic disc (Flechon *et al.* 2004). Unpublished results from our research group indicate that SSEA-1 is exclusively expressed in the porcine ICM at Day 6, remains restricted in the early epiblast at D8 and is downregulated in the late epiblast at Day 10, where expression is observed in the trophectoderm. Hence, it could be a potential marker of pESCs. Finally, SSEA1 has been detected in porcine PGCs (Takagi *et al.* 1997).

Finally, the tumor rejection antigens, TRA-1-60 and TRA-1-81, normally synthesized in undifferentiated cells, are used as markers for hESCs (Xu *et al.* 2001). However, in the pig, no reports on expression of TRA in ESC-like cells are available and they were not detected in PGCs (Takagi *et al.* 1997).

6.2.4. Other types of pluripotent stem cells

6.2.4.1. Epiblast stem cells

Recently, a new type of pluripotent stem cell termed epiblast stem cells (EpiSCs) was identified, which were derived from the epiblast of Day 6.5 and Day 7.5 mice and rats, respectively (Brons *et al.* 2007). EpiSCs are developmentally and functionally different from mESCs and can be maintained in chemically defined culture conditions including ACTIVIN and bFGF. They share many characteristics with hESCs such as expression of pluripotency markers and sensitivity to single-cell dissociation (Brons *et al.* 2007; Tesar *et al.* 2007). Although EpiSCs do exhibit capacity for multi-lineage differentiation and teratoma formation, they do not contribute significantly to chimeras (Tesar *et al.* 2007). Yet, reprogramming of EpiSCs to a mESC-like state has been achieved by overexpression of *Klf4* and the resulting cells were capable of producing germline chimeras (Guo *et al.* 2009). The findings outlined above demonstrate that the *Activin/Nodal* pathway plays a central role in pluripotency specifically representing the late epiblast cells just before gastrulation (Guo *et al.* 2009) and have lead to the theory that hESCs could in fact be EpiSCs rather than true ESCs capable of germline transmission (Rossant 2008).

6.2.4.2. Induced pluripotent stem cells

A significant breakthrough was made in the stem cell field when Takahashi and Yamanaka in 2006 reported the generation of induced pluripotent stem cells (iPSCs) from fetal and adult mouse fibroblast cells (miPSCs) by defined factors (Takahashi & Yamanaka 2006). The findings were based on the hypothesis that the oocyte and ESCs both contain factors required for the reprogramming and maintenance of pluripotency and the experimental setup comprised retroviral transduction of 24 candidate factors in different combinations into fibroblast cells. Finally, the factors were narrowed down to four candidates: *Oct4*, *Sox2*, *Klf4* and *C-myc* (Figure 7). The obtained miPSCs could be maintained on STO feeder cells in the presence of LIF and showed the same morphology as mESCs. In addition, miPSCs expressed the same pluripotency markers and were able to differentiate into cells of the three germ layers, which was demonstrated both in-vitro by EB formation and in-vivo by teratoma and chimera formation. Later, germline competent miPSCs were obtained (Wernig *et al.* 2007; Meissner *et al.* 2007) by eg. selection for reprogrammed cells expressing *Nanog* (Okita *et al.* 2007).

Another significant breakthrough was the generation of iPSCs from fetal and adult human fibroblast cells (hiPSCs), which could potentially overcome the need for destruction of a living human embryo (Takahashi *et al.* 2007; Yu *et al.* 2007; Park *et al.* 2008b). This was achieved using the same human factors *OCT4*, *SOX2*, *KLF4* and *C-MYC*, indicating that the essential reprogramming factors are conserved between these species. However, in one case, the *C-MYC* gene was successfully substituted with the transcription factor *LIN28* (Yu *et al.* 2007). Whereas miPSCs could be used as to study reprogramming or as a tool for studying disease mechanisms, hiPSCs hold great potential for use in cellular therapy of human diseases (Rolletschek & Wobus 2009). Firstly, these cells can be derived from a skin biopsy taken from a patient carrying a particular disease. These patient-specific hiPSCs could then be differentiated into mature cells and transplanted back into the same patient, thereby eliminating the risk of immunogenic rejection. Finally, hiPSCs could be used to study the mechanism of a particular disease or to test the effect of various drugs in-vitro. Recently, hiPSCs have been generated from a range of patients, including Parkinson's Disease (PD) and Huntington's Disease (HD) patients (Park *et al.* 2008a), which could be very useful in the future for in-vitro disease and transplantation studies. However, before hiPSCs can be used in cellular therapy, a number of safety issues has to be addressed. One of the most critical issues is that some of the reprogramming factors may be oncogenic (Takahashi *et al.* 2007) as reactivation of *C-myc* carried by a retrovirus resulted in tumor formation in ~20% of chimeric mice generated from miPSCs (Okita *et al.* 2007). However, it was later shown that *C-myc* could be excluded, which is an important step toward a safer application (Wernig *et al.* 2008a) and it was found that six of 37 chimeras derived from miPSCs died of tumors within 100 days after birth whereas all 26 chimeras generated without *C-myc* survived this period (Nakagawa *et al.* 2008). In addition, treatment with a histone deacetylase inhibitor called valproic acid enabled reprogramming of primary human fibroblasts with only the two factors *OCT4* and *SOX2* (Huangfu *et al.* 2008). Furthermore, it was found that human neural stem cells, which already express *SOX2*, *KLF4* and *C-MYC* endogenously, could more easily be reprogrammed by *OCT4* and *KLF4* (Kim *et al.* 2008b), or even by *OCT4* alone (Kim *et al.* 2009). Another important issue is the viral integration of factors, which may lead to continued expression of transgenes. However, reprogramming without integration in the genome has been accomplished by use of adenovirus (Stadtfield *et al.* 2008), plasmids (Okita *et al.* 2008), transposons (Kaji *et al.* 2009; Woltjen *et al.* 2009), small molecules (Silva *et al.* 2008) and even proteins (Zhou *et al.* 2009). Hence, during the last 3 to 4 years, this exciting technology has taken a giant step towards a clinical application.

Recently, porcine iPSCs (piPSCs) have also been generated, which may be very important for safety and efficacy testing prior to transplantation of hiPSCs to human patients (Roberts *et al.* 2009). This was achieved almost simultaneously by three different groups, which all relied on viral transduction of porcine fetal fibroblasts (PFFs) with the human factors *OCT4*, *SOX2*, *KLF4* and *C-MYC* (Ezashi *et al.* 2009; Wu *et al.* 2009), in addition to the corresponding mouse factors (Esteban *et al.* 2009). The morphology was reported to be similar to hESCs and primary porcine epiblast cells, with a high nucleus to cytoplasm ratio and prominent nucleoli (Wu *et al.* 2009). Like their human counterparts, piPSCs could be maintained on MEF feeder cells supplemented with bFGF for more than 220 population doublings (Ezashi *et al.* 2009), although one study did not use bFGF (Wu *et al.* 2009). Although they expressed the basic pluripotency markers, OCT4, NANOG and SOX2, a difference in the expression of surface markers was reported, as one group found expression of SSEA1, as in mESCs (Ezashi *et al.* 2009), whereas another group found expression of SSEA3 and 4 as well as TRA-1-60 and 1-81, as in hESCs (Wu *et al.* 2009). The pluripotency of piPSCs was, in all cases, confirmed using EB formation as well as teratoma formation. However, germ line chimeras, which is considered the final proof of pluripotency, was not shown. Given the recent reports on differences between ICM derived ESCs and epiblast-derived EpiSCs, generation of piPSCs in the presence of LIF is currently being pursued, which could turn out to hold germ line potential (Telugu *et al.* 2009). Studying the signaling network maintaining pluripotency in piPSCs could hold the key to establishment of pESCs in the future.

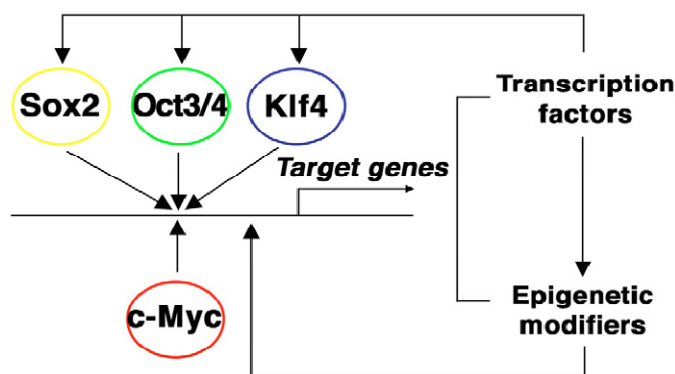


Figure 7. Establishment of pluripotency in somatic cell nuclei. The four transcription factors, *OCT4*, *Sox2*, *Klf4* and *C-myc*, were found to be sufficient to establish pluripotency in the nuclei of fibroblasts. *Oct4*, *Sox2* and *Klf4* might function together to activate target genes to establish the stable pluripotent transcription factor network, as well as the pluripotent epigenome, whereas *C-myc* might enhance the accessibility of target genes by stimulating DNA replication. (From Niwa *et al.* 2007).

6.3. Reporters in stem cell research

Three different types of reporters have been used in stem cell research to monitor the expression of a particular gene in response to certain stimuli. These include promoter constructs under the control of regulatory DNA, fusion constructs for localization and tracking of a protein in living cells and bicistronic gene constructs which allow for transcription of two functional proteins from the same promoter (Habermann *et al.* 2007). Initially, β -galactosidase and luciferase were the genes most frequently used to monitor transcription. The staining of β -galactosidase confers a strong signal intensity and this method has been used for the initial characterization of the expression pattern of *Oct4* (Yeom *et al.* 1996) and *Nanog* (Mitsui *et al.* 2003) in murine embryos. In contrast, luciferase was most often used to monitor quantitative transcription and has played an important role in determining the regulatory elements in both the *Oct4* (Okumura-Nakanishi *et al.* 2005) and *Nanog* (Wu & Yao 2005; Hattori *et al.* 2007) upstream promoters. However, these reporters are not suitable for measuring promoter activity at different time-points due to the need for enzymatic substrates and fixation (Soboleski *et al.* 2005).

6.3.1. Green fluorescent protein as a reporter

To date, Green Fluorescent Protein (GFP) is the most utilized reporter protein, due to its independence from enzymatic substrates, which makes it particularly promising in transgenic embryos and animals (Tsien 1998). GFP was first discovered in 1962 in the jellyfish *Aequorea victoria* (SHIMOMURA *et al.* 1962), however, it was not until the protein was cloned and sequenced (Prasher *et al.* 1992) and inserted in other species (Chalfie *et al.* 1994; Inouye & Tsuji 1994), that its potential as a reporter was realized. Reportedly, the protein consists of 11 β -strands forming a hollow cylinder in which an α -helix bearing the chromophore is located (Tsien 1998). The barrel structure protects the chromophore from degradation, and the chromophore, which consists of the three amino acids Serine, Tyrosine and Glycine, can undergo spontaneous oxidation, thereby emitting fluorescence (Tsien 1998).

Reportedly, three separate elements can determine if a fluorescent protein is suited as a reporter. Firstly, it must have a strong signal intensity. Secondly, it should not interfere with the cellular physiology and finally, it should have a short biological half-life (Habermann *et al.* 2007). In 1995, a red shifted variant of GFP, called enhanced GFP (EGFP), was identified and isolated by site directed mutagenesis (Heim *et al.* 1995; Cormack *et al.* 1996). This protein exhibited a 100 fold in-

creased fluorescence intensity, as well as improved folding compared to wild type GFP. Later, a “humanized” EGFP was generated by codon optimization (Yang *et al.* 1996) and by inclusion of a ribosome binding site (Kozak 1989), which allowed the protein to be expressed in eukaryotic cells. To improve the half-life of EGFP, a destabilized version, termed dEGFP, has also been generated. The gene mouse ornithine decarboxylase (*Modc*) was found to contain a PEST sequence (rich in proline, glutamine, serine and threonine) in its C-terminus, which targets it for rapid degradation by the proteasome (Li *et al.* 1998). This PEST sequence has been added to the EGFP variants, thereby lowering the half-life of EGFP from around 24 hours to two hours. Short half-lives are reported to be especially suited for the study of promoter activity and the protein exhibits lower cytotoxicity due to reduced accumulation (Habermann *et al.* 2007).

6.3.1.1. GFP reporters in embryos and stem cells

There are numerous reports on the use of EGFP as a reporter during early embryonic development as well as in embryonic- and adult stem cells. In 1995, it was demonstrated that EGFP could function as a reporter in transgenic mouse pre-implantation embryos (Ikawa *et al.* 1995). Later, it was found that porcine SCNT embryos containing EGFP also expressed the protein in all cells and were able to develop to the blastocyst stage (Uhm *et al.* 2000). EGFP can be used to measure quantitative gene expression, as the intensity of GFP fluorescence is directly proportional to mRNA abundance (Soboleski *et al.* 2005). This feature was exploited in a study by Wuensch and co-workers in which EGFP was used to quantitatively monitor expression of *OCT4* in bovine SCNT embryos (Wuensch *et al.* 2007). Hadjantonakis and co-workers have also shown that EGFP is functional in mESCs, and that these cells can be used to generate transgenic mice containing EGFP (Hadjantonakis *et al.* 1998). In the human, a EGFP reporter of the pluripotency gene, *REX1* was shown to be an efficient tool to monitor the undifferentiated state of hESCs (Eiges *et al.* 2001). Furthermore, recent advances in generation of iPSCs (Takahashi & Yamanaka 2006) has relied on the use of pluripotency reporters, such as the *Oct4* and *Nanog* promoters linked to EGFP, for selection of reprogrammed colonies (Okita *et al.* 2007). EGFP has also been used in different types of adult stem cells. Wang and colleagues fused the tubulin promoter to EGFP and used it to isolate neural precursor cells from murine and avian brains (Wang *et al.* 1998). Ohtsuka and colleagues have furthermore shown, that a destabilized EGFP coupled to the promoter of the proliferation promoting genes *HES1* and *HES5*, could be used to accurately detect and localize neural stem cells in the murine brain (Ohtsuka *et al.* 2006).

6.3.1.2. Other types of fluorescent proteins

Since the discovery of GFP, a range of different fluorescent proteins have been isolated and optimized for different purposes. Many of these, such as yellow and blue fluorescent proteins, are derived directly from GFP by amino acid substitutions (Tsien 1998). However, others have been isolated from different types of marine animals. The first red fluorescent protein called DsRed was isolated from the coral *Discosoma* (Matz *et al.* 1999) and was later optimized for expression in mammalian cells (DsRed2 and DsRed express). Recently, several fluorescent proteins were isolated from the evolutionary distinct *Copepoda* species, which is a type of zooplankton. One of these proteins, called TurboGFP showed a faster maturation than EGFP and brighter fluorescence in *Xenopus* embryos (Evdokimov *et al.* 2006). Like EGFP, this protein consists of β -barrels, although is formed of tetramers instead of a dimers (Figure 8). A red fluorescent protein (RFP) called TurboRFP was furthermore isolated from a sea anemone which possesses fast maturation and superior intensity compared to DsRed2 (Merzlyak *et al.* 2007). In addition, both of these proteins have been fused to a PEST sequence to obtain destabilized versions, which should be particularly suitable for promoter studies and the generation of transgenic animals. When Bell and colleagues compared TurboRFP to other fluorescent proteins, they found that in contrast to DsRed, it showed strong fluorescence which was maintained after fixation (Bell *et al.* 2007). However, the fluorescence was reported to differ from tissue to tissue.

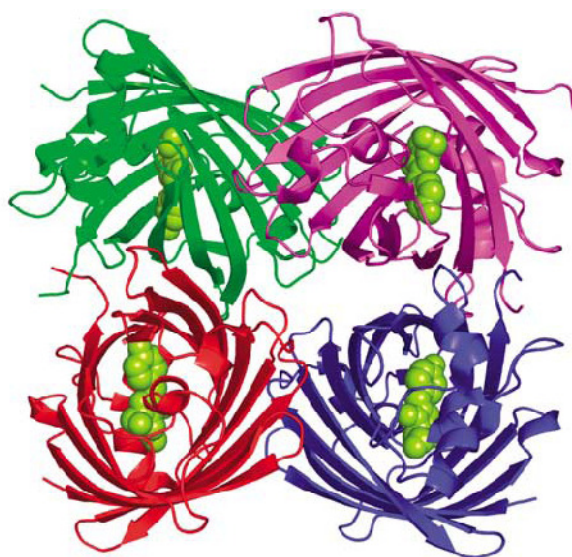


Figure 8. Crystal structure of TurboGFP. Like other green fluorescent proteins, the structure of TurboGFP is a β -barrel. It consists of four identical monomers (green, violet, red and blue) which forms a tetramer structure. The chromophore is shown as green van der Waals spheres. (From Evdokimov *et al.* 2006).

6.3.2. Transgenic methods in the pig.

There are several different ways of creating transgenic individuals in livestock species. One way is by injection of exogenous DNA directly into the pro-nuclei of oocytes or zygotes, which has been used to analyze the function of a murine *Oct4* reporter in porcine and bovine embryos (Kirchhof *et al.* 2000), however, in this method the expression is reported to be mosaic and integration is often less than 1% (Habermann *et al.* 2007). Viral integration of DNA has also been attempted, however, the risk of silencing of the transgene should be considered when using this technique.

Another method is sperm mediated gene transfer (SMGT) (Lavitrano *et al.* 1989), in which exogenous DNA is incubated with a sperm cell and taken up by passive osmosis, thereby entering the nuclei. When the sperm cell is injected into a matured oocyte, it develops as an in-vitro fertilized embryo carrying a stably integrated transgene. The versatility of this method has been demonstrated by generation of transgenic pigs containing three different fluorescent proteins (Webster *et al.* 2005). However, a downside of this method is that the integration sites are unknown and the amount of gene inserts may fluctuate between individual embryos and experiments (Habermann *et al.* 2007).

The final method is somatic cell nuclear transfer (SCNT) (Niemann & Kues 2000; Wolf *et al.* 2000), in which a transgene is introduced into a mature cell, such as a fibroblast cell, by transfection, electroporation, nucleofection or viral integration. After selection of colonies carrying the transgene by use of antibiotics, single cells are injected into enucleated oocytes (devoid of maternal DNA), fused by an electric current, activated chemically or by electric current and allowed to reprogram in medium that prevents cell cycle progression and cultured in-vitro (Niemann *et al.* 2003). Compared to SMGT, this method is more reproducible, as a single cell can be expanded and used for SCNT (Habermann *et al.* 2007). The downside is that only a small number of cloned embryos develop normally as they are unpredictably affected by epigenetic reprogramming. However, an increased need for transgenic pigs for biomedical research has resulted in extensive focus on the optimization of SCNT (Vajta 2007). A particular method of SCNT called hand-made cloning, which does not require the need for micromanipulation (Vajta *et al.* 2001), has been used to produce transgenic blastocysts expressing EGFP (Kragh *et al.* 2004).

Several groups have generated transgenic pigs by means of SCNT, hand-made cloning or SMGT containing fluorescent cell markers, which are of particular interest for xeno-transplantation and disease modelling (Matsunari & Nagashima 2009). However, only 2 out of 17 studies, have used other fluorescent proteins than EGFP to produce transgenic pigs and all relied on the use of constitutive promoters whereas inclusion of regulatory promoter regions has not been described (Matsunari & Nagashima 2009).

6.4. Neural progenitor cells

Replacement of damaged neurons by cell transplantation is actively being explored as a potential treatment for many neurodegenerative diseases such as Alzheimer's Disease (AD) and Parkinson's Disease (PD). Since the first isolation of neural stem cells (NSCs) 20 years ago (Temple 1989), these cells have provided new hopes in this field. However, an understanding of the signaling pathways controlling neural specification is crucial in order to exploit their therapeutic potential. In the literature, neural progenitor cells (NPCs) are often used as a synonym of NSCs, although the former is more restricted in its proliferation potential. In the following section, the cells will be referred to as NPCs.

6.4.1. Multipotency and self-renewal

In general, NPCs are defined by two functional traits: Their ability to self-renew as well as their multipotency, which means the ability to generate cells capable of producing neurons, astrocytes, and oligodendrocytes (Gage 2000). However, NPCs can also be unipotent, giving rise to only a single type of neural cell. Additionally, the proliferation and differentiation of NPCs is controlled by specialized microenvironments (Basak & Taylor 2009). Hence, NPCs are not just one type of cell, but are constantly subject to positional and temporal changes in the brain (Temple 2001). This is illustrated by NPCs from the ventral midbrain, which are the only type of NPCs capable of generating functional mature dopaminergic neurons in-vivo (Conti *et al.* 2006).

6.4.1.1. NPC niches and function

In general, all cells in the adult brain originate from dividing NPCs (Fish *et al.* 2008). The earliest NPCs found in the embryo are the neuroepithelial cells of the neural plate, as they later give rise to all the neurons of the mammalian central nervous system (Hyttel P *et al.* 2010). In the fetal brain, NPCs are located in the ventricular zone (VZ) of the telencephalon (Conti *et al.* 2006) and later in the adult brain, NPCs are found in the subventricular zone (SVZ) of the lateral ventricle (Lewis 1968; Doetsch *et al.* 1999). From this region, neuroblasts migrate along the rostral migratory stream into the olfactory bulb (OB), where they finally differentiate into mature neurons (Corotto *et al.* 1993). In addition, NPCs can be found in the subgranular zone (SGZ) of the hippocampal dentate gyrus, where they primarily differentiate into granule cells (Kaplan & Bell 1983; Palmer *et al.* 1997), which has been shown to be crucial for spatial learning and memory (Imayoshi *et al.* 2008).

NPCs can undergo symmetrical division, resulting in the generation of two new daughter cells (Figure 9A). This occurs when the mitotic spindle is organized parallelly to the apical (inner) surface of the neural plate or the SVZ and SGZ (Fish *et al.* 2008). In contrast, asymmetrical division of NPCs occurs when the spindle is located perpendicularly to the apical surface (Figure 9B). The result is the formation of an inner NPC which remains mitotic, as well as an outer post-mitotic neuron. Alternatively, asymmetrical division can generate a migrating neuroblast precursor cell which later gives rise to two post-mitotic neurons (Figure 9C). In general, it is believed that neurogenesis precedes gliogenesis in mammals (Temple 2001).

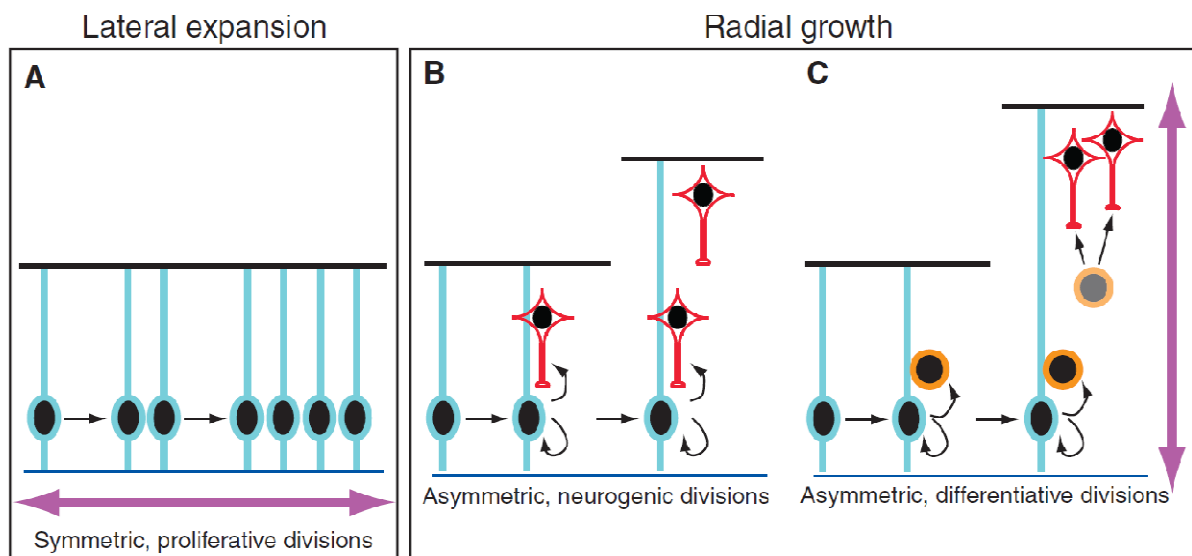


Figure 9. The mode of neural progenitor cell (NPC) division and its effect on the direction of cortical expansion and growth (lateral expansion versus radial growth). (A) Lateral expansion (horizontal arrow), in which one NPC generates two NPC daughter cells, occurs as a result of the symmetric, proliferative division (blue). (B,C) Radial growth (vertical arrow) occurs as a result of either (B) asymmetric, neurogenic divisions of NPC (blue), in which one NPC generates one NPC as well as one neuron (red), or (C) asymmetric, differentiative divisions of NPCs (blue), in which one NPC generates one NPC and one neuroblast (orange), which in turn generates two neurons (red) and is thereby consumed (dimmed orange) (From Fish *et al.* 2008).

6.4.1.2. Derivation of NPCs

20 years ago, NPCs were first isolated from the fetal forebrain of rats and mice (mNPCs) and cultured in-vitro (Temple 1989; Cattaneo & McKay 1990; Reynolds *et al.* 1992). These cells were able to differentiate into mature neurons in-vitro. Neural progenitor cells have also been derived from the adult rat and mice SVZ and SGZ (Palmer *et al.* 1995), as well as from other areas of the brain, such as the retina (Trobepe *et al.* 2000), the OB (Pagano *et al.* 2000) and the spinal cord (Shihabuddin *et al.* 2000). Initially, NPCs were cultured as neurospheres, which consists of a mixture of NPCs and adult neural cells (Conti *et al.* 2006). However, transplantation of neurospheres was reported to be problematic due to their heterogeneous nature. Later, it was found that NPCs could be cultured in homogeneous populations in adherent monolayer culture (Ying & Smith 2003; Conti *et al.* 2005). Furthermore, NPCs have been derived from mESC in-vitro (Okabe *et al.* 1996), which is commonly achieved by treatment of EBs with retinoic acid (RA) (Gajovic *et al.* 1997).

In human, NPCs (hNPCs) have been derived from fetal brains (Carpenter *et al.* 1999) as well as post-mortem adult brains (Svendsen *et al.* 1999). These cells show a capacity to proliferate for more than one year in-vitro. Furthermore, hESCs have been used as a source of NPCs. In general, three different methods have been used to promote neural induction from hESCs. The most common method is based on spontaneous differentiation, by maintaining hESC cultures without passage or by use of EB formation (Reubinoff *et al.* 2001). This method commonly results in generation of rosette structures, resembling neuroepithelial cells of the embryonic neural tube. (Denham & Dotti 2009). The second most common approach is co-culture of hESCs with murine stromal cells, such as PA6 or MS5 cells, which also results in rosette structures (Perrier *et al.* 2004). Finally, Chambers and colleagues have recently shown neural differentiation of hESCs and hiPSCs in a defined manner by directly inhibiting the *BMP* and *SMAD* signaling pathways with the two inhibitors, NOGGIN and SB431542 in adherent monolayer culture (Chambers *et al.* 2009). The hNPCs were capable of generating both dopamine- and motoneurons in-vitro.

In the pig, only a few studies have reported the generation of NPCs (pNPCs). Porcine NPCs have been derived from fetal brains (Armstrong *et al.* 2001; Armstrong *et al.* 2002; Harrower *et al.* 2006), which, when cultured in-vitro, showed a capacity to differentiate into both neurons and glia. Recently, isolation of pNPCs from the SVZ of adult pigs has been reported, which were able to maintain multipotency and proliferation for up to 6 passages (Liard *et al.* 2009). In contrast to

mouse and human, pNPCs have not been derived from ESCs due to the lack of validated pESC lines. However, Puy and co-workers recently derived pNPCs from ICM cells of porcine blastocysts, which could differentiate into glial cell types in-vitro (Du *et al.* 2009).

6.4.2. Signaling pathways of NPCs

In the telencephalon, where most NPCs reside, the patterning mechanisms from the early neural tube, including FGF, SHH, WNT, and BMPs appear to be conserved (Corbin *et al.* 2008). In rodents, it has been shown that proliferation of NPCs in the SVZ increased after bFGF and epidermal growth factor (EGF) administration in-vivo, with EGF having a more dramatic effect (Kuhn *et al.* 1997). EGF (Morrison *et al.* 1987) and bFGF (Nurcombe *et al.* 1993) have also been shown to maintain long-term in-vitro proliferation of NPCs isolated from the SVZ. In contrast, NPCs residing in the SGZ were unaffected by in-vivo supplementation of either EGF or bFGF (Kuhn *et al.* 1997) but seem to proliferate in response to bFGF in-vitro (Ray *et al.* 1993). It has been shown that the competence of NPCs to generate neurons, astrocytes, and oligodendrocytes reflects a deregulation of dorso-ventral patterning, which is caused by exposure to bFGF during in-vitro culture (Gabay *et al.* 2003). Hence, bFGF seems to play a role in resetting NPCs to an undifferentiated state (Pollard *et al.* 2008). Furthermore, LIF has been shown to stimulate proliferation of NPCs in-vivo (Bauer & Patterson 2006) and promotes long propagation in-vitro (Andersen *et al.* 2008). In addition to these mitogens, several other signaling pathways have been shown to be involved in maintaining the undifferentiated stage of NPCs, including *Shh* (Ahn & Joyner 2005), *Notch* (ndroutsellis-Theotokis *et al.* 2006) and *Wnt* signaling (Lie *et al.* 2005).

Due to ethical concerns, studies on the regulation of hNPCs has mostly been carried out in-vitro. In general, many of the signals and mechanisms involved in neural induction seems to be highly conserved across species (Denham & Dottori 2009). Human NPC media are also commonly supplemented with bFGF and EGF (Dottori & Pera 2008) and hNPCs have also been shown to be regulated by *WNT* (Davidson *et al.* 2007), *SHH* and *NOTCH* signaling (Elkabatz *et al.* 2008). However, there are also fundamental differences between hNPCs and mNPCs. One example is RA treatment, which appears to be involved in directing hNPCs towards spinal cord progenitors (Li *et al.* 2005). Furthermore, studies have shown differences between key neural pathways controlling fetal and hESC derived hNPCs (Shin *et al.* 2007), as well as fetal and adult brain-derived hNPCs (Maisel *et al.* 2007).

The regulation of pNPCs has not been subject to detailed studies, although they have been shown to respond to the same mitogens such as bFGF and EGF (Armstrong *et al.* 2002).

6.4.3. NPC markers

To date, no unique markers of NPCs have been identified. Instead, researchers rely on a multitude of markers, such as the intermediate filament *Nestin*, the transcription factors *Sox2*, *Pax6*, the RNA binding protein *Musashi* and the neural cell adhesion molecule (*Ncam*) (Schwartz *et al.* 2005). In general, these markers seem to be quite conserved between species.

Neuroepithelial stem cell protein (*Nestin*) is a class six intermediate filament protein expressed by NPCs located in the VZ and the SVZ (Lendahl *et al.* 1990; Doetsch *et al.* 1997). *Nestin* is considered a useful, although imperfect, marker of NPCs as it can be expressed by early neurons as well (Schwartz *et al.* 2005). In the pig, *NESTIN* expression has been reported in NPC populations cultured from the adult porcine brain (Armstrong *et al.* 2002; Uchida *et al.* 2003) and in ICM-derived NPCs (Du *et al.* 2009).

Sox2 is a transcription factor of pluripotent cells (see: pluripotency markers) as well as NPCs. At the beginning of neurogenesis, *Sox2* expression becomes restricted to the neural plate (Papanayotou *et al.* 2008) and through embryogenesis it is expressed in NPCs residing in the VZ of the developing neural tube and in the SVZ and SGZ of the adult brain (Catena *et al.* 2004). Moreover, constitutive expression of *Sox2* is reported to inhibit neuronal differentiation and results in the maintenance of progenitor characteristics (Graham *et al.* 2003). *Sox2* is expressed in brain-derived mNPCs and hNPCs (Graham *et al.* 2003) as well as in fetal brain derived pNPCs (Schwartz *et al.* 2005).

Paired box 6 (*Pax6*) is a transcription factor used as an early marker of neuroectodermal differentiation (Callaerts *et al.* 1997). In the fetal brain it is expressed in the VZ, the developing eye, the OB epithelium and in the spinal cord (Walther & Gruss 1991) and has been reported to be expressed by mNPC and hNPC (Suter *et al.* 2009). *Pax6* is also known to directly modulate *Sox2*, thereby controlling the expression of NPCs in the SVZ (Wen *et al.* 2008). In the pig, *PAX6* has been shown to be expressed in retinal progenitor cells (Klassen *et al.* 2007) but not in fetal- and ICM-derived pNPCs (Schwartz *et al.* 2005; Du *et al.* 2009).

Other important markers of hNPCs and mNPCs, including the RNA binding protein *Musashi1* (Kaneke *et al.* 2000) and the neural cell adhesion molecule (*Ncam*) (Maisel *et al.* 2007), have also been reported to be expressed by fetal- and ICM-derived pNPCs (Schwartz *et al.* 2005; Du *et al.* 2009). In contrast, the marker *p75* Neurotrophin R (*p75*), which is expressed by human, mouse and bovine neural crest progenitor cells (Lazzari *et al.* 2006; Lee *et al.* 2007), has not been reported to be expressed in the pig.

6.5. Neural stem cell therapy

A range of severe neurodegenerative diseases are currently affecting millions of people worldwide. Due to the remarkable complexity of the brain, many of these diseases are incurable with existing medical treatments. Instead, therapies rely on prolonging life, by administration of various drugs which may inhibit cell death and/or promote survival of remaining healthy cells. However, in the case of PD, cell based therapy has been shown to constitute a realistic alternative and trials using primary fetal tissue for transplantation has been completed in more than 300 patients (Lindvall & Bjorklund 2004). In contrast to fetal tissue, ESCs could provide an inexhaustible and more ethically acceptable source of cells for cell based treatment (Conti *et al.* 2006). Yet, as ESCs are known to cause teratomas in-vivo, in-vitro differentiation into NPCs or mature neurons is necessary prior to transplantation. In this respect, large animals, such as the pig, may prove to be essential for evaluating the risks and causes.

6.5.1. Neurodegenerative diseases

Parkinson's Disease is a progressive neurodegenerative disease, affecting 1 in a 100 people over 65 years, with as many as 50,000 new cases each year (Dauer & Przedborski 2003). The primary pathological feature is the death of dopamine producing neurons located in the substantia nigra in the brain (Freed 2002). The outcome is a lack of inhibition of motor neurons in the striatum, resulting in excessive muscle contraction, which gives rise to symptoms such as tremor and rigidity.

Alzheimer's Disease is the most common form of degenerative dementia affecting mainly elderly people and leading to progressive symptoms of memory loss (Selkoe 1999). The pathology includes degeneration of cholinergic cells in the cerebral cortex due to extracellular deposition of amyloid- β protein, plaques and neurofibrillary tangles (Auld *et al.* 2002).

Other severe neurodegenerative diseases includes HD, which is an inherent disease affecting mainly the striatum of the brain (Walker 2007), Multiple Sclerosis (MS) which affects the formation of myelin sheaths and leads to progressive deterioration of neurological function (Lublin & Reingold 1996) and Amyotrophic Lateral Sclerosis (ALS) which is a disease with the pathology of degeneration of motoneurons leading to muscle weakness and atrophy (Al-Chalabi & Leigh 2000).

6.5.2. Animal models of neurodegenerative diseases

Within the field of neurodegenerative diseases, the rodent has traditionally been the model of choice (Poncelet *et al.* 2009). However, large animal models often provide an essential bridge between insights into fundamental biology and the realities of treating a human disease (Wakeman *et al.* 2006). This is illustrated by several studies in which PD patients transplanted with human fetal derived tissues developed dyskinesia, whereas in the rat, these cells showed functional integration (Hagell *et al.* 2002). Hence, the rodent may not be the most optimal animal model of human neurological diseases. While monkeys are considered an adequate model in neurotransplantation, economical issues as well as ethical concerns limit the use of this animal in practice (Wakeman *et al.* 2006). In contrast, the pig has been suggested as an excellent candidate of neurodegenerative diseases by several authors (Vodicka *et al.* 2005; Wakeman *et al.* 2006; Lind *et al.* 2007; Poncelet *et al.* 2009). This is due to their gyrencephalic brain, which in contrast to the lissencephalic brain of rodents, more closely resembles the human brain (Hofman 1985). In addition, pigs are easily bred, have a long life span (12–15 years), produce large litter size and have a short gestation period (113–115 days) compared to monkeys (Lind *et al.* 2007). The minipig constitutes a particularly interesting model due to its relatively large brain and similar physiological and pathophysiological responses compared to humans (Wakeman *et al.* 2006) and while the use of inbred animals in experiments generally causes diminished variability (Lind *et al.* 2007). Important models of PD, HD and MS have already been generated in the minipig, and recently, an AD minipig was generated by means of SCNT (Kragh *et al.* 2009).

6.5.3. Stem cell transplantation in neurodegenerative disease models

To date, most transplantation studies have been carried out with rodents in which a PD-like condition is induced by injection of the chemical 6-Hydroxydopamine (Conti *et al.* 2006) into the substantia nigra of one hemisphere. However, transplantation of cells into a complex organ like the brain is not a trivial task and depends on several issues such as the affected area, as well as the type of disease and its pathology. In PD, in which a paracrine network of dopamine producing cells is involved, the most common strategy is transplantation directly to the affected site, the striatum (Conti *et al.* 2006). Identifying the correct type of cells for transplantation is another major task, which often includes a tradeoff between the use of NPCs, maintaining high plasticity but with the risk of incorrect incorporation, and more specialized neurons with lower plasticity.

6.5.3.1. Stem cell transplantation in Parkinson's Disease models

Transplantation of undifferentiated mESC into parkinsonian rats has been attempted, however, despite generation of functional dopaminergic neurons, some of these animals developed fatal teratomas (Bjorklund *et al.* 2002). This study made it clear that neural induction in addition to elimination of undifferentiated cells prior to transplantation is required. Kim and colleagues applied a genetic approach in which the nuclear receptor related 1 (*Nurr1*) promoter was used to select tyrosine hydroxylase (TH) expressing cells derived from mESCs prior to transplantation into parkinsonian rats (Kim *et al.* 2002). Interestingly, behavioral studies showed a significant reduction in motor asymmetry in the treated rats without evident teratoma formation. The therapeutic potential of iPSCs has also been demonstrated in-vivo as miPSC derived NPCs were able to functionally integrate into the murine brain (Wernig *et al.* 2008b). Additionally, dopaminergic neurons generated from miPSCs were able to improve behaviour in a rat model of PD.

Recently, the focus has turned to the use of hESCs for transplantation in animal models of PD. Roy and co-workers, Geeta and co-workers, and Yang and co-workers all showed long-term functional recovery of rodent models of PD using dopaminergic neurons derived from hESC by coculture, EB formation, or adherent culture, respectively (Roy *et al.* 2006; Geeta *et al.* 2008; Yang *et al.* 2008). Such studies show that hESC derived dopaminergic cells retain the same therapeutic capacity as shown in the mouse.

To date, most studies on transplantation of pNPCs have relied on the rodent PD models mentioned above. Armstrong and co-workers have transplanted pNPCs derived from fetal pigs in a rat model of PD (Armstrong *et al.* 2001; Armstrong *et al.* 2002), however, very few TH positive neurons were generated, and no functional recovery was observed. Later it was found that fetal derived pNPCs could form long distance axons and synapses with rat neurons (Uchida *et al.* 2003) and Harrower and co-workers found that fetal derived pNPC maintained long term survival in a rat model of PD when subjected to extended in-vitro culture prior to transplantation (Harrower *et al.* 2006).

6.5.3.2. Stem cell transplantation in other types of disease models

In contrast to PD, which involves paracrine signaling in which even a partial pattern repair may lead to a significant functional recovery (Conti *et al.* 2006), studies on NPC transplantation in other types of animal disease models such as AD, HD and ALS are more complicated due to the wide-

spread pathology of these diseases. However, NPC transplantation may be beneficial via the release of molecules that may either stimulate the regenerative potential or increase the survival of neurons (Conti *et al.* 2006). This is illustrated by a recent study, in which transplantation of mNPCs derived from newborn mice were able to improve cognition in a transgenic mouse model of AD through stimulation of brain-derived neurotrophic factor (BDNF) (Blurton-Jones *et al.* 2009). A superior target of cell replacement with limited requirements of pattern repair is represented by diseases characterized by glial degeneration, such as MS (Conti *et al.* 2006). It was shown that mESC-derived oligodendrocytes migrated into the host tissue, produced myelin and myelinated host axons in a rat model of MS, established by chemical demyelination of the spinal cord (Liu *et al.* 2000). In the pig, transplantation of oligodendrocytes to a rat model of MS has also been attempted (Smith & Blakemore 2000). In this study, it was found that pNPCs required commitment to the oligodendrocyte lineage prior to transplantation in order to achieve significant remyelination.

With the establishment of pNPCs and the emergence of important porcine disease models of PD, HD and AD (Lind *et al.* 2007), the pig could constitute an excellent large animal model of allogeneic stem cell therapy of human neurodegenerative diseases.

7. Materials, methods and results

A detailed description of the materials and methods and results are presented in manuscripts I to III.

7.1. Manuscript I. OCT4 expression in outgrowth colonies derived from porcine inner cell masses and epiblasts

Title

OCT4 expression in outgrowth colonies derived from porcine inner cell masses and epiblasts

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Abstract

The present study was conducted to test different methods for porcine inner cell mass (ICM) and epiblast isolation and to evaluate the morphology and expression of pluripotency genes in ICM- and epiblast-derived outgrowth colonies (OCs) and passages thereof with particular attention on the relationship between *OCT4* expression and embryonic stem cell (ESC)-like morphology. A total of 104 zona pellucida-enclosed and 101 hatched blastocysts were subjected to four different methods of ICM and epiblast isolation, respectively: Manual isolation, immunosurgery, immunosurgery with manual cleaning, or whole blastocyst culture. OCs were established on mouse embryonic fibroblast (MEF) cells and categorized according to morphology and OCT4 staining. Though all isolation methods resulted in ESC-like OCs, immunosurgery with manual cleaning yielded significantly higher rates of ICM/epiblast attachment and subsequent ESC-like morphology, whereas, no significant difference was found between ICM and epiblasts with respect to these characteristics. All ESC-like OCs showed nuclear OCT4 staining and expression of *OCT4*, *NANOG* and *SOX2* as evaluated by RT-PCR. Upon initial passages, the expression of pluripotency markers was, however, gradually lost in spite of maintained ESC-like morphology. In conclusion, we have established a robust system for derivation of ESC-like OCs from porcine ICM and epiblasts and we have shown that localization of OCT4 is associated with an ESC-like morphology although this relationship is lost during early passages.

Introduction

Embryonic stem cells (ESCs) are usually derived from the inner cell mass (ICM) of preimplantation embryos. ESCs have the capacity of infinite self-renewal and can, under the proper conditions, differentiate into all cell types of the body. The successful isolation and culture of ESCs has been reported first in mouse (Evans and Kaufman 1981; Martin 1981) and later in human (Thomson et al. 1998). In the mouse, it has later been demonstrated that the ICM and epiblast give rise to two different stem cell populations, ESCs and epiblast stem cells (EpiSCs), respectively, (Vallier et al. 2009). Interestingly, mouse EpiSCs share more similarities with human ESCs than mouse ESCs do. Attempts to establish ESCs or EpiSCs from other mammals, including the large domestic species, have been reported repetitively, but ultimate pluripotency has only been demonstrated by differentiation into both somatic and germ cell lineages in mouse and rat (Pease and Williams 1990; Kondoh et al. 1999; Buehr et al. 2008; Li et al. 2008).

To realise the use of ESCs or EpiSCs as a therapeutic tool in man it is of great importance to develop an animal model more closely related to humans than mice and rats. The pig offer great advantages in this respect due to anatomical and physiological similarities as well as phylogenetic proximity to human. In the pig, the ICM differentiates into epiblast and hypoblast around the time of hatching on Day 6-7 of gestation, and until around Day 10, the epiblast is covered by trophoblast, i.e. Rauber's layer (Oestrup et al. 2009). Hence, stem cell isolation from pre-hatching embryos is considered to give rise to ESC-like OCs while isolation from post-hatching embryos is considered to give rise to EpiSC-like OCs.

Over the past two decades many attempts to establish porcine ESC or EpiSC lines have been reported (Evans et al. 1990; Notarianni et al. 1990; Piedrahita et al. 1990; Strojek et al. 1990; Hochereau-de Reviers and Perreau 1993; Talbot et al. 1993; Anderson et al. 1994; Moore and Piedrahita 1997; Wianny et al. 1997; Chen et al. 1999; Miyoshi et al. 2000; Li et al. 2003; Li et al. 2004; Li et al. 2004; Brevini et al. 2007). However, no germ line transmission has yet been described, characterization with respect to pluripotency markers has been sparse, and the ESC-like cell lines have not proven to renew indefinitely. Hence, the basic conditions for establishment of porcine ESCs or EpiSCs are still to be defined.

An issue of great importance when culturing ESCs and EpiSCs is a proper characterization. In addition to morphology, characterization must be based on the expression of certain markers such as the transcription factors OCT4, SOX2, and NANOG, surface molecules as the stage specific embryonic antigens (SSEA1, 3 and 4) and the tumour rejection antigens (TRA1-60 and TRA 1-81). However, species differences exist with respect to expression patterns: For example, murine ESC express SSEA1 and -3 while human express SSEA3 and -4 (Ginis et al. 2004). It still needs to be clearly defined which of these markers porcine ESC-like cells express.

The transcription factor OCT4 is considered the gatekeeper of pluripotency in mouse and man (Niwa et al. 2000) and is exclusively expressed in the ICM at the blastocyst stage in these species. In the pig, on the other hand, *OCT4* is expressed in both the ICM and the trophectoderm of the blastocyst before hatching (Kirchhof et al. 2000). Later, in the hatched blastocyst, controversy exists with respect to the distribution of *OCT4* expression: In one investigation the presence of the protein was exclusively demonstrated in the epiblast (Vejlsted et al. 2006), whereas, another indicates the presence of OCT4 in the trophectoderm as late as Day 11 (Keefer et al. 2007). These controversial findings call for a further investigation of trophodermal OCT4 expression after hatching of the porcine embryo. In addition, a previous report indicated inconsistent expression of *OCT4* in porcine ESC-like cells (Brevini et al. 2007). Hence, a closer investigation of the relationship between ESC-like morphology and OCT4 localization in porcine ESC-like cells is also required.

Along these lines, the present study set out (1) to clarify the expression pattern of OCT4 in porcine blastocysts after hatching, (2) to optimize the methodology for isolation of porcine ICMs and epiblasts and establishment of OCs thereof, and (3) to investigate the morphology of porcine ESC-like cells in relation to the expression of pluripotency markers with special emphasis on OCT4.

Materials and Methods

Unless otherwise stated, materials were purchased from Sigma-Aldrich, Broendby, Denmark.

Feeder cells

Mouse embryonic fibroblast cells (MEF; kindly donated from Hagedorn Research Institute, Gentofte, Denmark) were prepared from whole mouse embryos collected at 13-14 days gestation, expanded, Mitomycin C treated and frozen at -80 °C. One day prior to an ESC experiment, MEF cells were thawed in a 37°C warm water bath, re-suspended in medium containing Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum (FBS, Invitrogen, Taastrup, Denmark), 1% glutamine and 1% penicillin/streptomycin and cultured in 3.5 cm petri dishes (Nunc, Roskilde, Denmark) at 37°C in a density of $1 \times 10^4/\text{cm}^2$.

Flushing of embryos

Female genital organs from sows (Danish landrace x Yorkshire crosses) collected at a local abattoir 5-8 days post insemination were transported to the laboratory in a 37 °C warm water bath for a maximum period of one hour. Each uterine horn was flushed with 150 ml embryo flushing medium (LIFE Pharmacy, KU, Frederiksberg, Denmark) with 1% FBS, and embryos were isolated by stereo-microscopy. Selected embryos at the blastocyst stage were transferred to DMEM containing 10% FBS for further processing. Overall, 27 sows were slaughtered yielding a total of 393 embryos of which 205 of the highest graded embryos were used for in-vitro culture, whereas the rest were fixed for stainings.

Isolation of inner cell masses (ICM) and epiblasts

The zona pelucida was removed from non-hatched blastocysts by submission to 5mg/ml pronase in DMEM low glucose (Invitrogen, Taastrup, Denmark) with 0.1% polyvinyl alcohol (PVA). The blastocysts were subsequently divided into four groups assigned for different methods of ICM/epiblast isolation: (1) manual isolation (ICM:10; epiblasts:18) where the ICM/epiblast was microscopically isolated from the trophectoderm using insulin needles; (2) immunosurgery (ICM:20; epiblasts:20) where blastocysts were incubated for 10 min in 10% guinea pig anti-pig-serum (kindly provided by Tiziana Brevini, Center for Stem Cell Research, University of Milan, Italy) in DMEM low glucose with 0.1 % PVA followed by treatment with 10% guinea pig complement (kindly provided by Tiziana Brevini) in DMEM low glucose with 0.1 % PVA until the tro-

phectoderm was visibly affected; (3) *immunosurgery with manual cleaning* (ICM:11; epiblasts:40) where the former two methods were combined in order to obtain a cleaner ICM/epiblast; or (4) *whole blastocyst culture* (ICM-embryos:63; epiblast-embryos:23) where intact blastocysts were directly subjected to culture.

Culture of OCs and passages

For establishment of OCs, isolated ICMs, epiblasts and whole blastocysts were cultured at 38°C on MEF cells in porcine ES medium consisting of DMEM low glucose supplemented with 40% Hams F10 (Invitrogen, Taastrup, Denmark), 10% knock-out serum replacement (KSR; Invitrogen, Taastrup, Denmark), 5% FBS, 1% nucleotide stock, 1% non essential amino acids (NEAA), 1% glutamine, 1% penicillin/streptomycin, 1% beta-mercaptoethanol (Invitrogen, Taastrup, Denmark) and 0.1% murine leukaemia inhibitory factor (mLIF; Chemicon, Hessen, Germany). At the initiation of culture, the medium was changed after 2 to 4 days and afterwards every second day. The rate of attached ICMs and epiblasts forming OCs was evaluated by phase contrast microscopy on Day 4 of culture.

OCs presenting ESC-like cells, i.e. closely apposed cells with high nucleus:cytoplasmic ratio and one or two distinct nucleoli, were subjected to physical passages onto fresh feeder cells by cutting of ESC-like areas into 5-10 small clumps using insulin needles and pipetting. Subsequent passages were performed approximately once per week.

Morphology and OCT4 staining

All OCs and passaged colonies were evaluated morphologically by phase contrast microscopy. Selected OCs (Days 7-9) and passaged colonies were processed for OCT4 immunostaining. Hatched Day 9 blastocysts (N=5) collected by the same procedure as described above were, in parallel, processed for staining in order to detect the specificity of OCT4 localization in the embryo.

Colonies were fixed in 4% paraformaldehyde (VWR, Herlev, Denmark) in 0.1M PBS for 15-30 min at room temperature and stored in 1% paraformaldehyde in 0.1M PBS at 5°C. Immunostaining of colonies comprised 30 min permeabilization in 1% triton X + 0.25% bovine serum albumin (BSA) in PBS followed by 30 min elimination of endogenous peroxidase by 3% hydrogen peroxide (H₂O₂; Merck, Glostrup, Denmark) in PBS, 3 times 5 min treatment with boiling 0.01M citrate buffer, pH 6

(LIFE Pharmacy, KU, Frederiksberg, Denmark) and 10 min blocking of unspecific antigen sites with DAKO Biotin Blocking System (Dako Denmark A/S, Glostrup, Denmark). Subsequently, the colonies were washed with PBS, incubated at 5°C over night with a primary antibody to OCT-3/4 (N-19 goat polyclonal SC-8628, Santa Cruz Biotechnology Inc., Santa Cruz, US) diluted 1:250 in 0.1% triton X + 0.25% BSA in PBS, washed 3 times 5 min in 0.1% triton X + 0.25% BSA in PBS and primary antibody binding was visualized by the secondary antibody kit Dako LSB®+System-HRP (Dako cytomation, Glostrup, Denmark) and AEC substrate kit (Zymed laboratories Inc., San Francisco, CA, USA). Finally, the specimens were counterstained with hematoxylin and examined using phase contrast microscopy.

Immunostaining of embryos comprised 2 hours incubation in 0.5% TNB blocking reagent (PerkinElmer, Hvidovre, Denmark) with 0.1% Triton X and 3% H₂O₂ followed by incubation over night at 4°C with primary antibodies OCT-3/4 (SC-8628) diluted 1:1000 and monoclonal mouse anti-E-cadherin (610181, BD Biosciences) diluted 1:1000 in 0.5% TNB with 0.1% Triton X. Embryos were washed 3 times 20 minutes in 0.1 M PBS with 0.1% Triton X and incubated for 2 hours with highly cross-absorbed secondary antibodies Cy2-anti-goat and Cy5-anti-mouse (Jackson ImmunoResearch Laboratories Inc., USA) diluted 1:500 in 0.5% TNB and 0.1% triton X. Finally, embryos were washed 3 times 20 minutes in 0.1 M PBS with 0.1% Triton X and stored in methanol at -20°C. Embryos were cleared in BABB (a 1:2 mixture of benzyl alcohol to benzyl benzoate) just prior to confocal microscopy. Specificity of OCT-3/4 antibody using blocking peptide was previously verified (Vejlsted et al. 2006).

RT-PCR

When passage of ESC-like colonies was performed, fragments were sampled and frozen in liquid nitrogen. RNA from colonies was purified using Dynabeads mRNA Direct micro kit (Dynal, Hamburg, Germany) following the manual supplied by the manufacturer and cDNA was synthesized immediately after using First strand cDNA synthesis kit (Fermentas, Copenhagen, Denmark) following the manual supplied by the manufacturer. RNA from porcine day 7 blastocysts served as positive control. PCR was performed using HotStarTaq DNA polymerase (Qiagen, Ballerup, Denmark) with primers for *OCT4*, *NANOG* and *SOX2* (Carlin et al. 2006). *GAPDH* (Kuijk et al. 2007) served as positive control and H₂O as negative control. The following PCR conditions were used: Hotstart at 95°C for 15 min, 35 cycles including denaturation at 94°C for 30 sec, annealing at 56°C

for *NANOG*, *SOX2* and *GAPDH* and 68°C for *OCT4* for 30 sec, and elongation at 72°C for 45 sec, finalized by elongation at 72°C for 10 min and incubation at 4°C. PCR products were run on a 1% agarose gel and visualized by staining with ethidium bromide.

Statistical analysis

The statistical analyses were performed using R (2005). The binary response variables describing prevalence of either attached OCs or ESC-like colonies were analyzed using binary logistic regression with embryonic developmental stage and isolation method as explanatory dependent variables. The polytomous response OC type was analyzed using multinomial logistic regression with embryonic developmental stage and isolation method as dependent variables.

Results

OCT4 staining in hatched blastocysts

Hatched blastocysts displayed nuclear localization of OCT4 in the epiblast, whereas, no staining was observed in the trophectoderm or hypoblast (Figure 1).

Categorization of OCs

On the basis of morphology and OCT4 staining, five different OC categories were defined: (1) Epiblast-like OCs presenting rounded, multilayered cell clusters with a compact epiblast-like structure with homogeneous nuclear OCT4 staining (Figure 2A, B). (2) Solitary ESC-like OCs, presenting a monolayer of ESC-like cells with homogeneous nuclear OCT4 staining surrounded by no or few flattened lipid-rich cells without OCT4 staining (Figure 2C, E, D). (3) Clearly delineated ESC-like OCs presenting a monolayer of ESC-like cells with a homogeneous or, in few cases, a heterogeneous nuclear OCT4 staining (Figure 2G, H) with a clear demarcation to a surrounding of numerous lipid-rich flattened cells without OCT4 staining (Figure 2F). (4) Poorly delineated ESC-like OCs presenting a monolayer of ESC-like cells with homogeneous nuclear OCT4 staining but with an unclear demarcation to a surrounding of numerous lipid-rich flattened cells without OCT4 staining (Figure 2I, J). (5) Differentiated OCs consisting of varying types of larger, flat cells without OCT4 staining in more or less well defined colonies (Figure 2K, L). OCs which successfully converted to monolayer culture and showed positive OCT4 staining including category 2 (solitary ESC-like OCs), 3 (clearly delineated ESC-like OCs) and 4 (poorly delineated ESC-like OCs) will collectively be referred to as ESC-like OCs.

The four different ICM/epiblast-isolation methods resulted in OCs of all five categories except for manual isolation and immunosurgery, which did not give rise to epiblast-like OCs (Table 1). The statistical evaluation demonstrated no significant differences between manual isolation, immunosurgery and whole blastocyst culture with respect to proportion of attached OCs as well as proportion of ESC-like OCs. However, immunosurgery with manual cleaning yielded a significantly higher proportion of attached OCs (69% versus 49%) as well as a significantly higher proportion of ESC-like OC (59% versus 32%) compared to the pooled data of the other three isolation methods. There was no significant difference between ICMs and epiblasts with respect to attachment rate and categories of OCs. However, epiblast-derived OCs tended to exhibit a more homogeneous distribu-

tion of OCT4 positive cells in the ESC-like colonies. RT-PCR showed expression of *OCT4*, *NANOG*, and *SOX2* in all examined ESC-like OCs as well as in epiblast-like OCs (Figure 3).

Characterization of passages

A total of 24 OCs obtained after immunosurgery with manual cleaning resulted in 18 (75%) passage 1 (P1) colonies. In 11, 6, 3 and 2 cases, these colonies were passed on to P2, P3, P4, and P5, respectively, before they went into quiescence or differentiation (Figure 4A, C). A total of 18 OCs obtained by whole blastocyst culture resulted in 11 (61%) P1 colonies and 4 P2 colonies, but only one continued to P7 before it went into quiescence. Only a few colonies displayed nuclear localization of OCT4 after the first passage despite maintaining an ESC-like morphology (Figure 4B, D). In addition, *OCT4*, *NANOG*, and *SOX2* expression, as monitored by RT-PCR, was lost at P1 or in few cases at P2, except for a few colonies in which *NANOG* and *SOX2* expression was maintained up to P3 and P6 (Figure 2).

Discussion

Previous conflicting data on *OCT4* expression in porcine blastocysts has cast doubt upon the use of OCT4 as a reliable pluripotency marker in the pig. It has been shown that OCT4 is expressed both in ICM and trophectoderm before hatching, followed by down-regulation in the trophectoderm after hatching (Vejlsted et al. 2006). Yet, another study has shown OCT4 staining in both the epiblast and trophectoderm of hatched blastocysts (Keefer et al. 2007). Reevaluated in our hands, however, hatched blastocysts exhibit OCT4 staining exclusively in the epiblast. In addition, analysis of numerous OCs derived from both ICMs and epiblasts clearly demonstrated that OCT4 staining correlated well with ESC-like morphology, in the sense that OCT4 stained cells always exhibited ESC-like morphology although it was possible to find colonies with ESC-like cell morphology which lacked OCT4 staining. The correlation between OCT4 and ESC-like morphology was, however, in most cases lost during passages. In human, the existence of two OCT4 isoforms, OCT4A and OCT4B, has been reported (Cauffman et al. 2006). Whereas OCT4A is associated with pluripotent cells, OCT4B is also expressed in non-pluripotent cells, and caution is required when selecting antibodies meant to be specific for OCT4A (Liedtke et al. 2008). The presence of two OCT4 isoforms in the pig could explain the contradicting reports of OCT4 expression in hatched blastocysts since the antibody used for detection of OCT4 by Vejlsted et al., 2006 and in this study recognizes a human isoform of OCT4 at 43-50 KDa which corresponds with the size of isoform A, whereas, the antibody used by Keefer et al., 2007 recognizes a human 265 aa isoform of OCT4 which corresponds to isoform B. However, further experiments are required to shed light on this issue in the pig.

In this study, we have tested several different ICM/epiblast-isolation methods which all produced ESC-like cultures. We found that the most reliable method was immunosurgery with manual cleaning of the ICM/epiblast, as this method produced not only the highest rate of OCs but also the highest proportion of OCs exhibiting ESC-like morphology. When colonies were subjected to passage, only few survived and colonies never exceeded seven passages. Evaluation of pluripotency markers at the mRNA level revealed a strong down-regulation of *OCT4* during the initial passages, even though many of the passaged colonies maintained an ESC-like morphology. In 2007, Brevini et al. (Brevini et al. 2007) reported, that the expression of *OCT4*, in contrast to that of *NANOG*, may vary from passage to passage in porcine ESC-like cells. In our hands, *OCT4* expression did not fluctuate,

but was simply lost during early passages, whereas *SOX2* and *NANOG* expression was found in a few colonies at later passages. Since *SOX2* is known to be expressed in neural stem cells, the expression of this marker may also be associated with the onset of neural differentiation which is considered the default pathway in mammals (Kim et al. 2007). In mouse ESC, *Nanog* has been reported to stabilize the state of pluripotency without being a strict requirement for this condition (Chambers et al. 2007). Whether the same applies to porcine ESC- or EpiSC-like cultures is not yet known.

In order to establish stable ESC lines, the ICM-cells need to transform and assume ESC characteristics including the potential of self-renewal, setting differentiation on hold. The ability to undergo this transformation differed between isolation methods. For instance, culture of whole blastocysts and immunosurgery with manual cleaning produced some epiblast-like OCs. We believe that these OCs are characterized by a poor ICM/epiblast to ESC transformation as the cell clusters attached and expanded but retained a morphology resembling the epiblast of the embryonic disc. Consequently, such OCs typically went into quiescence or gave rise to outgrowth of differentiated cells. Interestingly, culture of whole blastocysts, where the trophectoderm is left intact, gave rise to a high rate of solitary ESC-like OCs where ESC-like cells grew as a clean homogeneous cell population. On the other hand, when the ICM/epiblast was isolated by either manual dissection, immunosurgery or immunosurgery with manual cleaning the number of surrounding, flat cells expanded dramatically. This was an unexpected finding, as we are confident that our immunosurgery if not eliminated, then at least reduced the amount of trophectoderm. Hence, we believe that the flat surrounding cells in the clearly and poorly delineated ESC-like OCs represent cells derived from the ICM/epiblast; potentially of hypoblast nature.

The choice of feeder layer for the establishment of porcine ICM- or epiblast-derived colonies has been examined in several studies, but with varying conclusions (Strojek et al. 1990; Catena et al. 2004; Li et al. 2004; Kim et al. 2007). In at least two studies, STO feeder cells were found to support ICM attachment better than other feeder cell types (Piedrahita et al. 1990; Kim et al. 2007) while other authors found the opposite (Strojek et al. 1990; Li et al. 2004). In our hands, STO feeder cells resulted in very low rates of attachment (18%, n=66; data not shown) in comparison with MEF (50%, n=20; Table 1) when used under similar conditions. Moreover, the OCs derived on STO feeder cells showed a much higher tendency to differentiate (data not shown).

In conclusion, we have established a robust system for derivation of ESC-like OCs from porcine blastocysts at the ICM and epiblast stage of development and have demonstrated expression of the pluripotency factors *OCT4*, *NANOG* and *SOX2* in the OCs. Moreover, cells with OCT4 localization presented a typical ESC-like morphology. During the initial passages, however, the cells lost the expression of first *OCT4* and, later, *NANOG* despite retaining an ESC-like morphology.

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Figure legends.

Figure 1: Hatched Day 9 blastocyst stained for OCT4 and E-cadherin. (A) Staining for E-cadherin. (B) Staining for OCT4. Note that the OCT4 staining is specific for nuclei in the epiblast. (C) Merge of A and B. Scale bars represent 50 μm .

Figure 2: Phase contrast and OCT4 immunostainings of porcine OCs. (A, B) Epiblast-like OC. The homogeneous nuclear OCT4 staining is obscured due to the thickness and compactness of the specimen (B). (C, D, E) Solitary ESC-like OC. Note the ESC-like (ESCL) morphology (C, E), the distinct nucleoli (arrowhead in E), the homogeneous nuclear OCT4 staining of the ESCL cells (D), and the lipid droplets of the surrounding flat cells (arrowhead in D). (F, G, H) Clearly delineated ESCL OC. Note the clear demarcation (arrowhead in F) between the ESCL area and the surrounding flat cells and either a homogeneous (G) or heterogeneous (H) nuclear OCT4 staining. (I, J) Poorly delineated ESCL OC. Note the unclear demarcation between the ESCL area and the surrounding cells (I) and the homogeneous nuclear OCT4 staining in the ESCL area (J). (K, L) Differentiated OC. Note the flat loosely packed cells (K) with lack of OCT4 staining (L). Scale bars represent 100 μm .

Figure 3: Characterization of OC and passages by RT-PCR. The figure shows an OC and a passage 3 colony examined for the expression of the pluripotency markers *OCT4*, *NANOG* and *SOX2* with *GAPDH* as reference. D7 blastocysts served as positive control tissue and H₂O as negative control. All samples were run on a 1% agarose gel and stained with ethidium bromide.

Figure 4: Phase contrast and OCT4 immunostainings of passages from porcine ESC-like OCs. (A, B) Passage 1 (P1) colony with ESC-like morphology (A) and nuclear OCT4 staining (B). (C, D) P4 colony with ESC-like morphology (C), but lack of OCT4 staining (D). Scale bars represent 100 μm .

Table 1: Distribution of blastocysts on ICM/epiblast-isolation methods and characterization of OCs.

ICM/epiblast isolation method	Number of embryos	Number of OCs (%) ^A	Colony Category					ESC-like (%) ^A
			Epiblast-like	Solitary ESC-like	Clearly delineated ESC-like	Poorly delineated ESC-like	Differentiated	
1 Manual isolation	28	13 (46%)	0	2	3	3	5	8 (29%)
2 Immunosurgery	40	21 (53%)	0	5	2	8	6	15 (38%)
3 Whole blastocyst culture	86	41 (48%)	5	15	5	6	10	26 (30%)
Combined (1-3)		75 (49%) ^a [41-57%]						49 (32%) ^a [25-39%]
4 Immunosurgery with manual cleaning	51	35 (69%) ^b [55-80%]	2	3	15	12	3	30 (59%) ^b [45-72%]

^A Percentage of embryos cultured.

Figures with different superscripts within columns are significantly different (P<0.05) and 95% confidence intervals are presented in square brackets.

Figure 1.

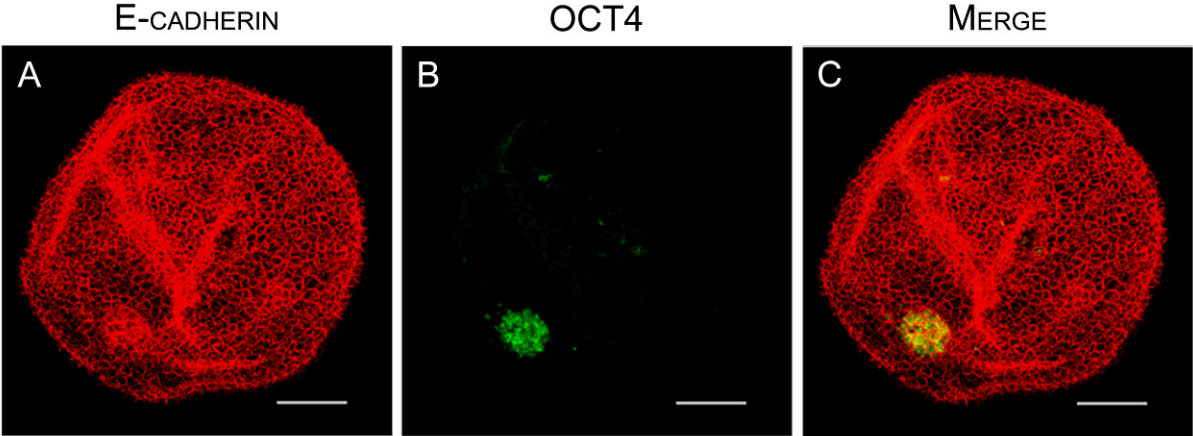


Figure 2.

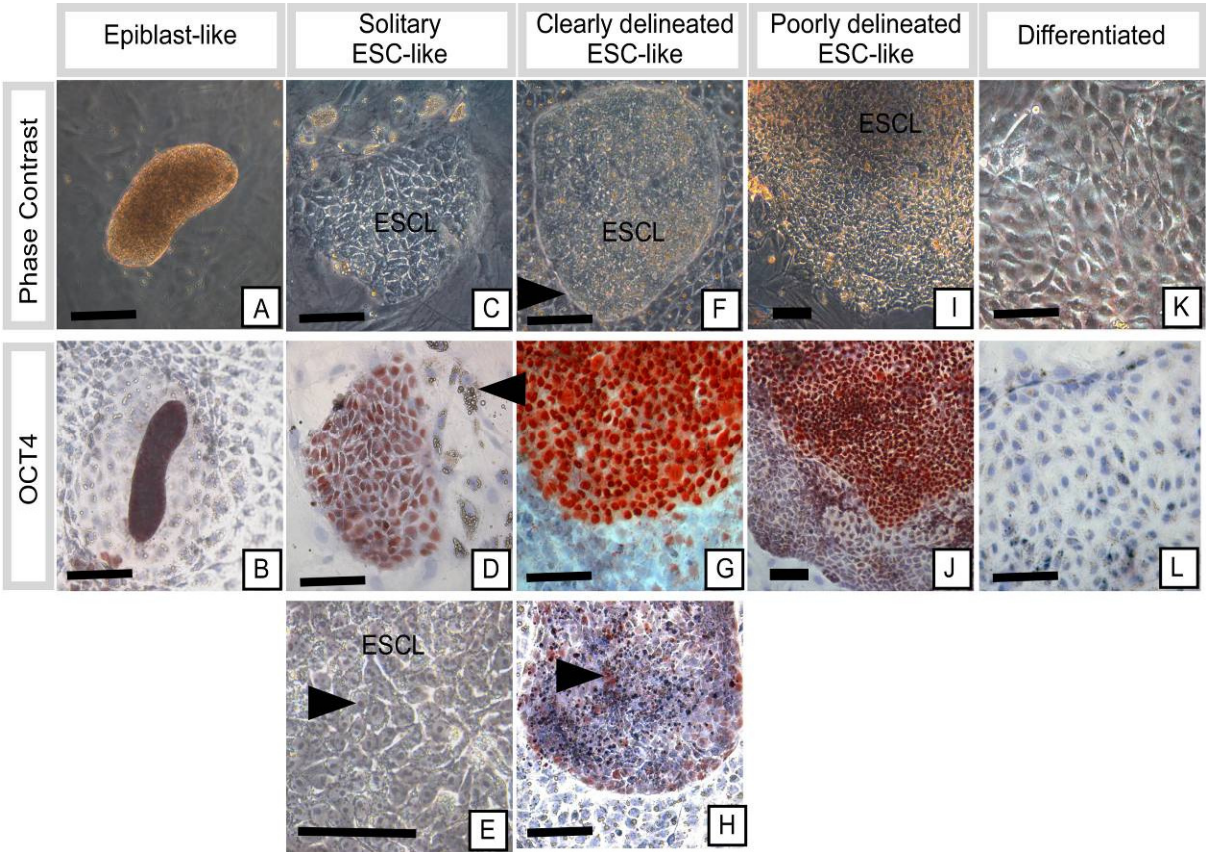


Figure 3.

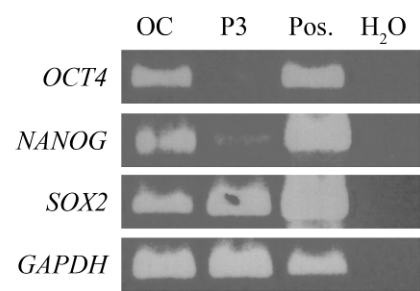
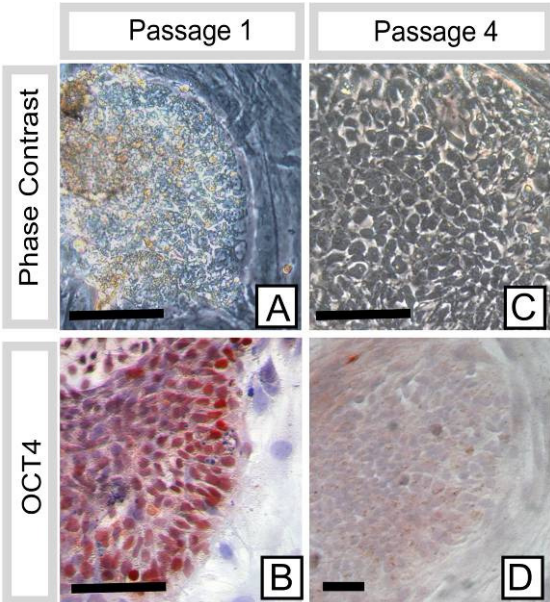


Figure 4.



7.2. Manuscript II. Analysis of porcine OCT4 and NANOG promoter regions and expression in pluripotent cells and transgenic embryos

Title: Analysis of porcine *OCT4* and *NANOG* promoter regions and expression in pluripotent cells and transgenic embryos.

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Running Title: Analysis of the porcine *OCT4* and *NANOG* promoter regions.

Abstract

Generation of a pluripotent reporter pig, containing reporter constructs of *OCT4* and *NANOG*, could constitute an important large animal model to study pluripotent populations in-vivo and for derivation of embryonic stem cell (ESC) and induced pluripotent stem cell cultures in-vitro. In this study, alignments of the porcine *OCT4* and *NANOG* promoter regions with homologous regions of other mammals have revealed important conserved transcription factor binding sites, including SP1/SP3, OCT4, SOX2, KLF4 and SMADs. Two *OCT4* reporter constructs were generated, each containing 6.6 kb porcine *OCT4* promoter sequence, including the distal enhancer (DE) which is shown to be active in ESCs and embryonic germ cells in the mouse, in front of a destabilized enhanced red fluorescent protein (pO4-dERFP-IRESpuro-pA) or an enhanced green fluorescent protein (pO4-EGFP). Furthermore, a *NANOG* reporter construct containing 2.2 kb porcine *NANOG* promoter sequence in front of a destabilized enhanced green fluorescent protein (pNG-dEGFP) was generated. Whereas pO4-dERFP-IRESpuro-pA was tested by transfection into mouse ESCs, the pO4-EGFP and NG-dEGFP reporter constructs were tested by attempting to produce transgenic embryos, using somatic cell nuclear transfer (SCNT) and sperm mediated gene transfer (SMGT). Low fluorescence was observed in mouse ESCs transfected with pO4-dERFP-IRESpuro-pA compared to a stably integrated murine *Oct4*-EGFP reporter construct. In contrast, the pO4-EGFP reporter construct showed expression in single blastomeres of 4-cell to morula stage embryos produced by SCNT and SMGT and NG-dEGFP reporter construct yielded ICM specific green fluorescence in blastocysts produced by SMGT, which points to an important role of *NANOG* in maintaining the pluripotent state in the pig. However, fluorescence was not observed in SCNT embryos containing NG-dEGFP. The lack of fluorescence in most cell types and transgenic embryos is likely due to a combination of factors, such as variation in endogenous *OCT4* expression, post-transcriptional modification as well as epigenetic silencing. Further studies are required to ensure correct expression of *OCT4* and *NANOG* reporter constructs before a pluripotent reporter pig can be established.

Introduction

Pluripotency is controlled by different signaling pathways in the mouse and human. Leukemia inhibitory factor (LIF) supports the undifferentiated state of mouse embryonic stem cells (mESCs) by activating the transcription factor STAT3 through the *Jak/Stat* pathway (Smith et al., 1988) and bone morphogenetic protein 4 (BMP4) can enhance self-renewal and pluripotency through the *Smad1/5/8* pathway (Ying et al., 2003). In contrast, in human embryonic stem cells (hESC), basic fibroblast growth factor (bFGF) is required for maintenance of pluripotency and self-renewal and ACTIVIN/NODAL signaling through *SMAD2/3* is necessary to maintain the pluripotent state (Xu et al., 2005; Vallier et al., 2005; James et al., 2005). Despite these signaling differences, octamer binding protein 4 (*Oct4*; also known as POU5F1), and *Nanog* are central downstream targets, which maintain the pluripotent state in both the mouse and human.

Oct4 maintains the pluripotency in the inner cell mass (ICM) by inhibiting the differentiation into the trophectodermal lineage (Niwa et al., 2000). In accordance, homozygotic *Oct4* knockout mice do not develop past the blastocyst stage due to the lack of a distinct ICM (Nichols et al., 1998). In addition, *Oct4* seems to be expressed in a quantitative manner, as downregulation of *Oct4* in mESCs has been shown to correlate with the formation of trophectoderm, whereas, upregulation results in formation of primitive endoderm (Niwa et al., 2000). Further evidence of a central role of *Oct4* in pluripotency has come from reprogramming of somatic cells to induced pluripotent stem cells (iPSCs), in which OCT4 was identified as one of the key reprogramming factors (Takahashi and Yamanaka, 2006). In contrast to the mouse, OCT4 is localized in both the ICM and trophectoderm of the unhatched human blastocyst (Cauffman et al., 2005) but is later found exclusively in the epiblast of the hatched blastocyst (Chen et al., 2009). It has been reported that *OCT4* splice variants are expressed differently in human pluripotent and non-pluripotent cells (Atlasi et al., 2008) and caution should be shown when using RT-PCR and immunocytochemical staining of OCT4 (Liedtke et al., 2008). Whereas OCT4A seem to be expressed in the nucleus, and is involved in maintaining pluripotency, OCT4B is only expressed in the cytoplasm of some individuals and does not seem to have an apparent function (Takeda et al., 1992; Cauffman et al., 2006). A retroposon called OCT4C has also been detected, which lacks introns and is not transcribed.

In the pig, OCT4 was found to be ubiquitously expressed prior to hatching (Kirchhof et al., 2000; Spencer et al., 2006; Kuijk et al., 2008; Keefer et al., 2007; Hall et al., 2009), which was also reported after hatching (Kirchhof et al., 2000; Kuijk et al., 2008). Hence, it was reasoned that *OCT4* is not a reliable pluripotency marker in the pig (Keefer et al., 2007). However, other studies have shown OCT4 protein localization exclusively in the epiblast after hatching (Vejlsted et al., 2006; Flechon et al., 2004; Vejlsted et al., 2006; Hall et al., 2009) and *OCT4* expression was detected in cultured ICM and epiblast cells but not in trophoblast cells (Blomberg et al., 2008). Around gastrulation, OCT4 becomes restricted posteriorly in the epiblast, after which it is found exclusively in primordial germ cells (PGCs) (Oestrup et al., 2009). Expression of endogenous *OCT4* in porcine iPSCs, which was downregulated upon differentiation, points to a significant role in maintaining pluripotency in the pig as well (Esteban et al., 2009; Ezashi et al., 2009; Wu et al., 2009). In contrast to the mouse and human, isoforms of OCT4 have not been reported in the pig.

The promoter of *Oct4* has been the subject of several studies and through alignments with promoter regions of different mammalian species such as mouse (Yeom et al., 1991), cow (van Eijk et al., 1999), human (Yang et al., 2005), and recently also rabbit (Kobolak et al., 2009), several conserved regions have been identified (Nordhoff et al., 2001; Yang et al., 2005). A distal enhancer (DE) is responsible for the expression of *Oct4* in the ICM and in PGCs, whereas, a proximal enhancer (PE) is active in the epiblast (Yeom et al., 1996). The former is of particular interest in pluripotent stem cell research, as it has been shown to be active in mESCs and embryonic germ cells (EGCs) as well (Ovitt and Scholer, 1998). The proximal promoter (PP), on the other hand, is required for general *Oct4* expression and downregulation upon differentiation. It has been shown that pluripotency is maintained through an autoregulatory network in which OCT4 binds to and upregulates the promoters of *Nanog* and *Sox2* (Okumura-Nakanishi et al., 2005). In addition, OCT4 acts in a negative feedback mechanism by binding to its own promoter, thereby limiting its own expression (Pan et al., 2006). In addition to regulation by transcription factors, the *Oct4* promoter region has been shown to be subject to epigenetic regulation. Furthermore, the DE, PE and the PP region were found to be hypomethylated in mESCs but hypermethylated in somatic cells, except for a binding site of transcription factors belonging to the SP1/SP3 family in the promoter region (Hattori et al., 2004). In addition, histone acetylation was also found to be involved in the regulation of *Oct4*.

Nanog expression is slightly later in murine pre-implantation development. The role of *Nanog* is to maintain the pluripotent state of the epiblast by inhibiting formation of the primitive endoderm (Mitsui et al., 2003). In accordance, *Nanog* null embryos did not develop beyond implantation, and the ICM failed to segregate and form the epiblast. Additionally, it has been shown that overexpression of *Nanog* can induce cytokine-independent self-renewal of undifferentiated mESCs (Chambers et al., 2003), whereas, downregulation of *Nanog* in mESCs results in primitive endoderm and trophoderm differentiation (Hyslop et al., 2005). However, a recent study has shown that mESCs containing a conditional *Nanog* knockout could be maintained in the undifferentiated state, despite being prone to differentiation (Chambers et al., 2007). It was concluded that *Nanog* is not essential for pluripotency, but acts as a safeguard against differentiation and mediates germ line potential. In human, NANOG is not identified prior to the expanded blastocyst stage where it is located in the early epiblast (Hyslop et al., 2005). Different splice variants of *NANOG* have been identified in both the mouse and human (Hart et al., 2004) and *NANOG* transcripts were reported to be present in several adult tissues as well.

Researchers have failed to detect NANOG by immunocytochemistry in porcine blastocysts prior to hatching (Kuijk et al., 2008; Hall et al., 2009; Blomberg et al., 2008). Yet, *NANOG* was detected in several other types of mature tissues by RT-PCR (Carlin et al., 2006; Blomberg et al., 2008). Like *OCT4*, it has been argued that *NANOG* is not a reliable pluripotency marker in the pig (Carlin et al., 2006; Kuijk et al., 2008; Blomberg et al., 2008). However, after hatching, NANOG was recently reported to be localized exclusively in the epiblast of Day 9-11 embryos (Hall et al., 2009) and thereafter is maintained exclusively in EGCs (Ostrup et al., 2009). Expression of endogenous *NANOG* in porcine iPSCs, which was downregulated upon differentiation, indicates a significant role in pluripotency in this species too (Esteban et al., 2009; Ezashi et al., 2009; Wu et al., 2009). In contrast to the mouse and human, isoforms of *NANOG* have not been detected in the pig. However, in other ungulates, the existence of *NANOG* isoforms have been reported and in the cow, NANOG A was localized in the epiblast, whereas, NANOG B was observed in polar trophoblast (Degrelle et al., 2005).

The proximal promoter (PP) region of *Nanog* is reported to consist of several conserved regions (Wu and Yao, 2005; Chan et al., 2009). Recently, it was shown that SMADs can bind directly to the *NANOG* promoter in hESCs (Xu et al., 2008). Binding of TGF β /Activin-responsive SMAD/2/3 was

shown to enhance the *NANOG* promoter activity in hESCs, whereas, binding of BMP4-responsive SMAD1/5/8 reduced the *NANOG* expression. Furthermore, in the mouse, it was found that SMAD1 binds directly to the *Nanog* promoter, which, in concert with STAT3 and Brachyury, play important roles in preventing mesoderm differentiation (Suzuki et al., 2006). These studies have helped to shed light on how NANOG sustains ESC self-renewal and controls differentiation in various species. However, in contrast to OCT4, NANOG is not required as a reprogramming factor of iPSCs but instead acts in the final step of reprogramming by instating ground state pluripotency (Silva et al., 2009). In the mouse, it was recently shown that OCT4 forms a complex with SOX2 and KLF4 and binds to the *Nanog* promoter, which is essential for reprogramming of somatic cells into iPSCs (Wei et al., 2009). Repression by DNA methylation and histone modifications has also been shown to play a role in the regulation of *Nanog* (O'Neill et al., 2006; Hattori et al., 2007). Two areas in the PP were found to be hypomethylated in ES cells but hypermethylated in somatic cells and while DNA methylation was most dominant in the proximal part of the PP, it was found that histone modification was dominant in the distal part of the PP.

Reporter constructs of *Oct4* and *Nanog* have been used to study the expression pattern of *Oct4* and *Nanog* in murine embryos (Yeom et al., 1996; Mitsui et al., 2003) as well as in mESC (Kuroda et al., 2005; Okumura-Nakanishi et al., 2005) and hESCs (Kuroda et al., 2005; Gerrard et al., 2005). A reporter construct containing the *Oct4* gene including 9 kb promoter sequence in front of an enhanced green fluorescent protein (EGFP) was used to monitor *Oct4* expression in murine pre-implantation embryos (Yoshimizu et al., 1999; Kirchhof et al., 2000; Szabo et al., 2002). Importantly, it was found that EGFP could be used as a measure of reprogramming of cloned blastocysts, which correlated with the frequency of mESC derivation (Boiani et al., 2002). The same *Oct4* reporter construct was later used to quantitatively monitor reprogramming in cloned bovine blastocysts (Wuensch et al., 2007) and was found to be expressed in porcine blastocysts as well (Kirchhof et al., 2000). A *Nanog* reporter construct has furthermore been used to monitor reprogramming of somatic cells to iPSCs (Okita et al., 2007; Maherali et al., 2007) resulting in germline-competent iPSCs with increased mESC-like gene expression (Okita et al., 2007). However, a recent study has indicated species specific variation in the regulation of pluripotency reporter constructs, as a human *OCT4* reporter construct was subject to epigenetic de-regulation in mouse embryonic fibroblast cells (Cha et al., 2008).

The regulatory parts of porcine *OCT4* and *NANOG* have not been subject to any detailed analyses. However, alignments with the promoter sequences of *OCT4* and *NANOG* with their homologs of other mammals could yield important insights into the regulation of pluripotency in the pig. Fluorescent reporter proteins have furthermore opened new vistas for embryology and stem cell research in livestock species (Habermann et al., 2007) and generation of a pluripotent reporter pig containing fluorescent reporter constructs of *OCT4* and *NANOG* could constitute an important large animal model to study pluripotent cell populations in-vivo as well as for derivation of ESCs and for selection of iPSCs in-vitro. However, to verify their function, fluorescent reporter constructs of *OCT4* and *NANOG* first need to be correlated with endogenous *OCT4* and *NANOG* expression in-vitro.

In the present study we have: 1) Isolated and analysed the promoter regions of porcine *OCT4* and *NANOG*; 2) Generated different fluorescent reporter constructs containing porcine-specific sequences of either *OCT4* or *NANOG* in front of red or green fluorescent proteins and; 3) Analysed the expression of the reporters in mESCs and porcine pre-implantation embryos generated by somatic cell nuclear transfer (SCNT) and sperm mediated gene transfer (SMGT).

Materials and methods

Unless otherwise stated, materials were purchased from Invitrogen, Carlsbad, CA.

Analysis of *OCT4* and *NANOG* promoter sequences

Bacterial artificial chromosomes (BACs) containing porcine *OCT4* and *NANOG* promoter sequences were identified by alignment with human homologous sequences (www.ensembl.org) (Hubbard et al., 2009). Alignments of *OCT4* and *NANOG* promoter sequences with other mammals, including macaque, human, cat, cow, mouse and rat, was carried out using Bioedit 7.0.4. Based on studies in the mouse and human, transcription factor binding sites were mapped according to the human transcriptional start site.

Generation of pluripotency reporter constructs

Generation of pO4dERFP-IRESpuro-pA

A BAC clone CH242-102G9 (Wellcome Trust Sanger Institute, Cambridge, UK), containing the porcine *OCT4* gene and promoter sequence, was purified using the Hispeed Plasmid Midi kit (Qiagen, Chatsworth, CA). Polymerase chain reaction (PCR) with the primers 5armEHf and 5armNf as well as 3armNf and 3armBr (Table 1) produced two products, called 5'- and 3'-arms, corresponding to the regions -6139 to -6575 and +19 to -340 relative to the transcriptional start site of the mouse *Oct4*, respectively (Okazawa et al., 1991). The 5'- and 3'-arm PCR products were digested with EcoRI/NheI and NheI/BglII respectively, and ligated into the EcoRI/BglII digested multiple cloning site (MCS) of the vector pTurboRFP-Dest1 (Evrogen, Moscow, Russia), in front of a destabilized enhanced red fluorescent protein (dERFP) using T4 DNA ligase (Fermentas, Burlington, ON) to produce pO4aRFP. pTurboRFP-Dest1 is reported to possess a fast maturity and strong intensity (Merzlyak et al., 2007) and due to the destabilization signal the reporter protein has a short half life of approximately 2 hours which is especially suited for the study of promoter activity (Habermann et al., 2007).

An internal ribosomal entry site gene linked to a puromycin selection gene (IRESpuro; Clontech, Ca, USA) and the poly-A tail of the bovine growth hormone (pA) were digested with NcoI/BamHI and SpeI/NcoI, respectively, and subcloned into XbaI/BamHI digested pBluescriptSK+ vector (pBSK; Agilent Technologies, CA, USA) to produce IRESpuro-pA.

A 1671 bp fragment of pO4aRFP containing the 5'- and 3'-arms and the dERFP was isolated by digestion with EcoRI/XbaI and ligated into NsiI/EcoRI digested IRESpuro-pA cassette in pBSK in conjunction with an XbaI/NsiI digested adapter, generated by hybridization of oligos XadN53 and XadN35 (Table 1), to generate pO4arep. A removable "lox2neo" cassette containing lox-P sites flanking the geneticin (G418) resistance gene was digested with HpaI/EcoRI and introduced into pO4arep by digestion with HpaI/EcoRI. The resulting vector was linearized with NheI and electro-transformed into recombineering competent *E. Coli* SW106, which had previously been electro-transformed with the BAC clone CH242-102G9 (Wellcome Trust Sanger Institute). Heat-induced recombineering occurred after 15 min incubation at 42°C, after which a final digestion with NotI/ClaI was carried out to isolate the 11.020 bp pO4dERFP-IRESpuro-pA from the pBSK backbone. The construct was digested with different combination of restriction enzymes and sequenced to verify correct ligation of fragments.

Generation of pO4-EGFP

High-fidelity PCR (Qiagen, Chatsworth, CA) with primers pO4RegF and pO4RegR (Table 1) was performed to amplify a region -6575 bp to +19 bp upstream of the transcriptional start site of *OCT4* using the BAC clone CH242-102G9 (Wellcome Trust Sanger Institute) as a template. The resulting PCR product, as well as the vector pmaxFP-Green-C (Lonza, Cologne, Germany), containing an enhanced green fluorescent protein (EGFP) (Evdokimov et al., 2006) were digested with AseI/AgeI and ligated, thereby exchanging the cytomegalovirus (CMV) promoter with the *OCT4* promoter region. Finally, the construct was linearized with AseI resulting in the 10.707 bp pO4-EGFP.

Generation of pNG-dEGFP

A BAC clone CH242-335 (Wellcome Trust Sanger Institute, Cambridge, UK) which aligned with the human *NANOG* gene and upstream sequence was used for generation of a *NANOG* reporter construct. However, no sequence information was available. Consequently, the BAC was purified with Hispeed Plasmid Midi kit (Qiagen, Chatsworth, CA), digested with different combinations of restriction enzymes and ligated into compatible ends of the pBSK vector. Following transformation into competent *E. coli* Top10 cells and incubation overnight at 37°C, plaque lifts were performed onto Hybond-N+ transfer membrane (GE Healthcare Bio-sciences, Little Chalfont, UK), according to the manufacturer's instructions. A 195 bp DNA probe recognizing exon 1 of the porcine *NANOG* gene was generated by PCR with the primers NG6f and NG200r (Table 1). The probe was used to

visualize colonies containing the *NANOG* gene and promoter sequence using Enhanced Chemiluminescence Direct labelling and Detection Systems (GE Healthcare Bio-sciences, Little Chalfont, UK), according to the manufacturer's instructions. Positive clones were digested with various combinations of restriction enzymes to determine their fragment size. In addition, terminal sequencing with T7 and M13 primers was performed and selected clones were fully sequenced and aligned to create a consensus sequence. Digestion with BamHI/PstI and BglII/PstI yielded two fragments, which were ligated into a BamHI digested MCS of pTurboGFP-N (Evrogen, Moscow, Russia), containing a dEGFP which possess the same characteristics as dERFP (Evdokimov et al., 2006). Finally, the CMV promoter was removed by digestion with BamHI/apaLI resulting in a 6076 bp pNG-GFP. The construct was verified by digestion with different combination of restriction enzymes and sequenced to verify correct ligation of fragments.

Transfection of mouse embryonic stem cells

Mouse embryonic stem cells containing a murine *Oct4*-GFP reporter construct were cultured according to Nichols and co-workers (Nichols et al., 1990). Briefly, mESCs were cultured overnight on gelatinized culture dishes in mESC medium consisting of GMEM medium (Sigma-aldrich, St. Louis, MO) supplemented with 2 mM glutamine (Invitrogen, Carlsbad, CA), 1 mM sodium pyruvate (Invitrogen), 1% nonessential amino acids (Invitrogen), 10% fetal bovine serum (FBS) (Invitrogen), 0,1% beta-mercaptoethanol (Sigma-aldrich, St. Louis, MO) and 20ng/ml leukemia inhibitory factor (Chemicon, Billerica, MA) at 37°C in 20% O₂, 5% CO₂ in N₂. Transfection of *Oct4*-GFP mESCs with pO4dERFP-IRESpuro-pA was carried out using Lipofectamine 2000, according to the manufacturer's instructions. Transfection with a vector containing a CMV promoter upstream of dERFP, and with H₂O was performed as a positive and a negative control, respectively. After four hours, the medium was changed to fresh mESC medium, and the cells were cultured for 24 hours at 37°C in 20% O₂, 5% CO₂ in N₂. The following day, the cells were fixed in 4% paraformaldehyde (PFA) for 20 min and transferred to 1% PFA. Fluorescence was observed using a Leica DMRB fluorescent microscope and Leica Application Suite version 2.81 (Leica Microsystems).

Somatic cell nuclear transfer

Nucleofection of porcine fetal fibroblast cells

Isolation and nucleofection of porcine fetal fibroblast cells (PFFs) was carried out according to Wuensch and colleagues (Wuensch et al., 2007). Briefly, PFFs were isolated from Day 27 embryos of the “Schwabisch Hällisch” landrace by use of collagenase and cultured in DMEM (Sigma-aldrich, St. Louis, MO) containing 5% FBS. At passage 3, the linearized reporter constructs pO4-EGFP and pNG-dEGFP were introduced into the PFFs by means of nucleofection with Amaxa basic nucleofector kit for primary fibroblasts using program U12 of the Amaxa nucleofection device (Lonza, Verviers, Belgium). For each construct, a total of 2.1 µg DNA was nucleofected into 0.8×10^6 PFFs which were selected in 0.6 mg/ml G418 for two weeks.

Somatic cell nuclear transfer

Ovaries were collected at a local abattoir and transported to the laboratory in phosphate buffered formalin (PBS) containing 75 µg/ml potassium penicillin G (Sigma-aldrich, St. Louis, MO), 50 µg/ml streptomycin sulfate (Sigma-aldrich) and 0.1% (w/v) polyvinylalcohol (PVA; Sigma-aldrich). Cumulus-oocyte complexes (COCs) were collected by aspiration and cultured in NCSU23 medium (Petters and Wells, 1993) supplemented with 0.6 mM cysteine, 10 ng/ml epidermal growth factor (EGF), 10% porcine follicular fluid, 75 µg/ml potassium penicillin G, 50 µg/ml streptomycin sulfate (all from Sigma-aldrich, St. Louis, MO) and 10 IU/ml equine chorionic gonadotropin (eCG) (Intervet, Millsboro, USA) and 10 IU/ml human chorionic gonadotropin (hCG) (Intervet). COCs were cultured for 22 h with eCG and hCG, and then without hormones for 20 h at 38.5°C in 20% O₂, 5% CO₂ in N₂. Oocytes displaying evenly granulated ooplasm and extrusion of the first polar body were selected for the experiments.

Somatic cell nuclear transfer was performed using in-vitro matured (IVM) oocytes as recipient cytoplasts, as previously described (Kurome et al., 2006). Enucleation was performed using a chemically assisted method (Yin et al., 2002). Oocytes were cultured in NCSU23 medium supplemented with 0.1 µg/ml demecolcine, 0.05 M sucrose and 4 mg/ml BSA (all from Sigma-aldrich, St. Louis, MO) for 0.5-1 h and then enucleated by aspirating the first polar body and adjacent cytoplasm in Hepes-TL-PVP containing 0.1 µg/ml demecolcine, 5 µg/ml cytochalasin B (CB) and 10% FBS (all from Sigma-aldrich, St. Louis, MO). Enucleation was confirmed by staining cytoplasts with 5µg/ml bisbenzimidazole (Hoechst 33342; Sigma-aldrich, St. Louis, MO).

A mixed population of PFFs containing stable integrations of either pO4-EGFP or pNG-dEGFP were used as nuclear donors after cell cycle synchronization by serum starvation for 48 h. A single donor cell was inserted into the perivitelline space of an enucleated oocyte. Donor cell-oocyte complexes were placed in a 280 mM mannitol solution (pH 7.2) containing 0.15 mM MgSO₄, 0.01% PVA, and 0.5 mM Hepes (all from Sigma-aldrich, St. Louis, MO) and held between 2 electrode needles. Membrane fusion was induced using an Electro cell fusion LF101 (NEPA GENE Co. Ltd., Chiba, Japan) by applying a single direct current (DC) pulse (200 V/mm, 20 μ s \times 1) and a pre- and post-pulse alternating current (AC) field of 5 V, 1 MHz for 5 s, respectively. Reconstructed embryos were cultured in NCSU23 for 1-1.5 h, followed by electrical activation. Reconstructed embryos were washed twice in an activation solution consisting of 0.3 M mannitol, 50 μ M CaCl₂, 100 μ M MgSO₄ and 0.01% PVA, then placed between 2 wire electrodes of a fusion chamber slide and overlaid with activation solution. A single DC pulse of 150 V/mm was applied for 100 μ s. Activated oocytes were treated with 5 μ g/ml CB for 3 h to suppress extrusion of the pseudo-second polar body. In-vitro culture of embryos was performed in 20- μ l droplets of Porcine zygote medium (PZM) (Yoshioka et al., 2002) in 5% O₂, 5% CO₂ in N₂ at 38.5 C.

Embryo transfer

Six to seven month old prepuberal gilts were used as recipients. Estrus synchronization was conducted by oral administration of altrenogest (Regumate, Serumwerk Bernburg, Germany) over a 15 day period, followed by intramuscular injection of 750 IU pregnant mare serum gonadotropin (Intergonan, Intervet, Germany) 24 hours after last gestagen administration. Ovulation was induced 3 days later by intramuscular injection of 750 IU human chorionic gonadotropin (Ovogest, Intervet, Germany). Two days after Intergonan treatment, recipients were anesthetized and brought into 45° dorsal recumbency. 1-2 cell-stage embryos containing pO4-EGFP or pNG-dEGFP were transferred laparoscopically into the right oviduct and embryos were cultured in-vivo for 10 days prior to flushing (Day 11-12 embryos).

Sperm mediated gene transfer

Ovary collection and in-vitro maturation

Danish Landrace x Yorkshire gilt ovaries were collected from a local abattoir (Danish Crown, Ringsted, Denmark) and were kept at 30 - 33°C from collection until follicular aspiration. The

COCs were aspirated and cultured in IVM medium containing TCM-199 (Sigma-aldrich, St. Louis, MO) supplemented with 50 ng/ml EGF (Sigma-aldrich, St. Louis, MO), 10 IU/ml eCG, 5 IU/ml hCG (Suigonan Vet., Intervet Scandinavia, Skovlunde, Denmark), 100 μ M 2-mercaptoethanol, 5 μ l/ml Insulin-Transferrin-Selenium, L-Glutamine-Penicillin-Streptomycin, and 4 mg/ml bovine serum albumin (BSA; Sigma-aldrich, St. Louis, MO) for 44 - 48 hrs at 38.5⁰C in 20% O₂, 5% CO₂ in N₂.

Preparation of semen

Semen from 10 Duroc boars was purchased from Hatting boar station (Hatting-KS, Ringsted, Denmark) as a mixed fresh ejaculate in extender and was kept at 16 - 18⁰C until use. Semen was washed free of extender twice in a non-capacitating medium (113 mM NaCl, 5 mM KCl, 5.56 mM glucose, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 22 mM Na-lactate, 5 μ g/ml phenol red, 50 μ g/ml gentamycine (all from Sigma-aldrich, St. Louis, MO)), then resuspended in capacitating IVF medium (90 mM NaCl, 12 mM KCl, 0.5 mM NaH₂PO₄, 25 mM NaHCO₃, 0.5 mM MgSO₄, 2 mM Na-pyruvate, 8 mM CaCl₂, 1.9 mM caffeine, L-Glutamine-Penicillin-Streptomycin, 5 μ g/ml phenol red, 4 mg/ml BSA (all from Sigma-aldrich, St. Louis, MO) and pre-incubated for 10 - 15 min at 38.5⁰C in 20% O₂, 5% CO₂ in N₂.

Sperm mediated gene transfer

From a final sperm concentration of 1 x 10⁶ cells/ml, 3 μ l was added to a 3 μ l 10% polyvinyl pyrrolidone droplet (PVP, Cook Medical, Bloomington, IN) with 3 μ l of either pO4-EGFP or pNG-dEGFP to a final conc. 4 ng/ml DNA and incubated at room temperature for one hour. H₂O served as a negative control. Prior to SMGT, mature oocytes were freed from cumulus cells by 5 – 10 sec of vortexing and individual sperm cells were immobilized by squeezing the tail between the bottom of the disc and the injection pipette (El Shourbagy et al., 2006). SMGT was carried out using a 25 μ m holding pipette and an 8 μ m injection pipette according to standard procedures. After SMGT, oocytes were cultured for up to 1 week in porcine zygote medium 3 (PZM-3) supplemented with 4 mg/ml BSA at 38.5⁰C in 5% O₂, 5% CO₂ in N₂ (Yoshioka et al., 2002).

Monitoring of fluorescence and fixation of embryos

Cleavage and blastocyst formation of SCNT and SMGT embryos was monitored over a 7 days culture period. Fluorescence microscopy was carried out at Day 2 (4-8 cell stage), Day 5 (morula

stage) and Day 7 (blastocyst stage). For immunocytochemical analysis, embryos from the same stages were fixed in 4% PFA for 20 min and stored in 1% PFA at 4°C. For reverse transcriptase PCR (RT-PCR), 5 SCNT blastocysts were pooled, transferred to lysis buffer (AH diagnostics, Oslo, Norway) and frozen at -80°C.

Flushing of somatic cell nuclear transfer embryos

Recipients were slaughtered at a local abattoir 10 days post embryo transfer (Day 11-12 embryos) and the uterus was isolated and transported to the laboratory in a 38°C warm water bath for a maximum period of one hour. Each uterine horn was flushed with 150 ml PBS containing 1% FBS, and embryos were isolated by stereo-microscopy. For immunocytochemical analysis, embryos were fixed in 4% Paraformaldehyde (PFA) for 20 min and stored in 1% PFA at 4°C. For reverse transcriptase PCR (RT-PCR), 5 embryos were pooled, transferred to lysis buffer and frozen at -80°C.

RNA purification

For reverse transcriptase PCR (RT-PCR), 5 SCNT blastocysts were pooled, transferred to lysis buffer (AH diagnostics, Oslo, Norway) and frozen at -80°C. The SCNT embryos were placed in lysis buffer (AH diagnostics, Oslo, Norway) and stored at -80°C. Total RNA was purified using the Nucleospin RNA XS kit (AH diagnostics) and reverse transcription was performed using RevertAid First strand cDNA synthesis kit (Fermentas, Burlington, ON) according to the manufacturer's instructions. For each sample, a negative reaction was included, by omission of M-MuLV Reverse Transcriptase enzyme.

PCR and RT-PCR

PCR was performed with primers of *OCT4* and *NANOG* (Table 1) with porcine chromosomal DNA as a positive control. RT-PCR was performed with primers of *GFP* (Table 1) with DNA from PFFs containing pO4-EGFP or pNG-dEGFP as positive controls and H₂O as a negative control. In both conditions PCR was carried out using Hot Start PCR master mix (Fermentas, Burlington, ON) with the following PCR conditions: Hotstart at 95°C for 15 min, 35 cycles including denaturation at 94°C for 30 sec, annealing at 56°C for 30 sec, and elongation at 72°C for 45 sec, and finalized by elongation at 72°C for 10 min and incubation at 4°C. The PCR and RT-PCR products were run on a 1% and a 3% agarose gel, respectively, and visualized by staining with ethidium bromide.

Immunocytochemistry

Immunocytochemistry of SCNT and SMGT embryos comprised 30 min permeabilization in 0,1% triton X + 0.25% bovine serum albumin (BSA) in PBS followed by 3 times 5 min treatment with boiling 0.01M citrate buffer, pH 6 (LIFE Pharmacy, KU, Frederiksberg, Denmark) and 30 min blocking of unspecific antigen sites with 5% normal donkey serum (Sigma-aldrich, St. Louis, MO) in PBS. Subsequently, the embryos were washed with PBS and incubated at 5°C overnight with primary antibodies anti-OCT4 goat polyclonal (SC-8628, Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:250 in 0.1% triton X + 0.25% BSA in PBS or anti-NANOG rabbit polyclonal (Pepro-tech, Rocky Hill, NJ) diluted 1:500 in 0.1% triton X + 0.25% BSA in PBS or anti-TurboGFP rabbit polyclonal (Evrogen, Moscow, Russia) diluted 1:1000 in 0.1% triton X + 0.25% BSA in PBS. The following day the embryos were washed 3 times in PBS, incubated 1 hour with fluorescent-conjugated secondary antibodies (Alexa Fluor) diluted 1:400 in 0.1% triton X + 0.25% BSA in PBS, washed 3 times in PBS, incubated in 0.1 µl/ml Bisbenzimidazole Hoechst (Sigma-aldrich, St. Louis, MO) and mounted in fluorescence mounting medium (Dako, Glostrup, Denmark). Specificity of OCT4 and NANOG antibodies has previously been verified (Vejlsted et al., 2006; Hall et al., 2009) and as a negative control, embryos were incubated in the absence of primary antibodies. All specimens were examined using a Leica DMRB fluorescent microscope and Leica Application Suite version 2.81 (Leica Microsystems).

Results

Alignment of the porcine *OCT4* gene and promoter region with other mammals.

The reported 5699 bp porcine *OCT4* coding sequence consists of five exons and is located on chromosome 7 (NM001886435) and the resulting mRNA sequence is 1083 bp (NM001113060). Alignment with bovine, human and murine *OCT4* showed that the 360 amino acid (aa) protein is extremely conserved between species as it shares 96,4%, 93,0% and 83,6% aa sequence identity among these species, respectively.

Several regions in the porcine *OCT4* reporter sequence showed homology with other mammals (Figure 1). A CCCTCCCCC sequence located -2065 to -2056 relative to the murine transcriptional start site was found to be 100% conserved between the other mammals. In addition, the sequence AGATGCATGACAAAG located -2020 to -2005 was also conserved except for a single nucleotide difference in the bovine and rat sequences. High sequence identity was also identified in the sequence TCCAGGCCCATTC AAGGGTTGAGCAC located -1047 to -1020 except for the mouse and rat which showed a few nucleotide differences. In addition, the sequence GGGGGCGGGGCCAGAGGTCAAGGCTA located -72 to -47 showed 100% similarity to the other mammalian sequences. Finally, several sequences with the consensus GGG(A/T)GGG or CCC(A/T)CCC were found to be highly conserved as well.

Alignment of the porcine *NANOG* gene and promoter region with other mammals.

The 6.487 kb porcine *NANOG* coding sequence consists of four exons and is located on chromosome 5 (EF522119) and the resulting mRNA sequence is 1181 bp (DQ447201). In contrast to *OCT4*, alignment with bovine, human and murine *NANOG* showed that the 304 aa protein is less conserved, as it shares 79,5%, 73,8% and 56,11% sequence identity among these species, respectively.

Homology was only observed in the *NANOG* PP region (Figure 2). The sequence TAAAAAGTGGAAACAAGGTGGACCTGCAA located -262 to -233 relative to the murine transcriptional start site showed sequence identity with other ungulates and primates, but differed from the rodents. In contrast, the sequence GTCTG located -175 to -171 was 100% conserved between the other mammals. The sequence TTTGCATTACAATG located -150 to -136 was also completely

conserved, except for a single nucleotide in the canine sequence. The sequences GGGGGTGTG and GGGGCGGGC located -83 to -74 and -64 to -55, respectively were conserved between the other mammals, however, some variations between primates, ungulates and rodents was observed. High sequence identity was also observed in a TATAA sequence located at -33 to -28, except in the rodents. Finally, several AGAC sequences located in the *NANOG* PP region were relatively conserved between other mammals.

Generation of a red fluorescent reporter construct of *OCT4*.

Three BAC clones with high homology to the human *OCT4* gene and promoter sequence were identified and PCR with primers of *OCT4* spanning exons 3 to 5 confirmed that all three clones contain the porcine *OCT4* gene as they produced a single product of the expected 898 bp (Figure 3A). In contrast, a reaction with chromosomal DNA produced an additional product of 268 bp, indicative of an intron-lacking pseudogene. The 189 kb BAC clone CH242-102G9, containing the complete *OCT4* gene as well as 53,066 kb upstream region was used in the present study, as it shows 99,6% sequence identity with the porcine *OCT4* coding sequence and 100% identity with the porcine *OCT4* mRNA sequence.

The *OCT4* reporter construct pO4dERFP-IRESpuro-pA was generated by homologous recombination with the BAC clone 242-102G9 to ensure the highest possible sequence identity (Figure 4A). It includes approximately 6.6 kb of the upstream region of *OCT4* inserted directly upstream of dERFP, The IRES sequence allows for a puromycin selection gene to be transcribed from the same messenger, which is useful for the selection of *OCT4* positive colonies, and a poly-A tail from the bovine growth hormone, ensures correct translation. The reporter construct also contains a constitutively expressed G418 resistance gene for selection of transfected cells, which is floxed with loxP sites for subsequent removal.

Generation of a green fluorescent reporter construct of *OCT4*

A second *OCT4* reporter construct called pO4-EGFP was generated by High-fidelity PCR (Figure 4B). It also consists of approximately 6.6 kb upstream region inserted in front of EGFP and includes a constitutive neomycin gene for selection of transfected cells.

Generation of a green fluorescent reporter construct of *NANOG*

Two BACs with homology to the human *NANOG* gene and promoter sequence were identified. PCR with primers of *NANOG* spanning exon 3 and 4 showed that the BAC clone CH242-335 produced a single product of the expected 300 bp, whereas, chromosomal DNA and another BAC clone produced an additional product of 160 bp indicative of an intron-lacking pseudogene (Figure 3B). A *NANOG* pseudogene has previously been identified on chromosome 1 (XM_001928688), which has a single exon of 1159 bp and lacks a poly-A-tail. It shares 99% sequence identity with the mRNA of the real *NANOG* gene.

Since sequence information was not available on BAC clone 242-335, colony blotting was used to generate the *NANOG* reporter construct pNG-dEGFP (Figure 4E). Hybridization with a *NANOG* probe (Figure 4C) and restriction digestion (Figure 4D) yielded a total of 40 clones containing inserts of varying size. Terminal sequencing with T7 and M13 primers identified that 7 of the clones contained exon 1 of the *NANOG* gene in either the 5' or 3' sequenced ends. Three of the largest clones were fully sequenced which resulted in a 2178 bp sequence with 100% sequence identity among them, which was inserted in front of dEGFP. The *NANOG* reporter construct includes a constitutive neomycin gene for selection of transfected cells. Recently, a sequence containing 3305 bp promoter sequence of the porcine *NANOG* gene was published (EF522119) (Miyamoto et al., 2008). The 2178 bp promoter sequence identified in this study matches this promoter sequence by 98%, indicating that the sequences are nearly identical.

***OCT4* reporter expression in mouse embryonic stem cells**

Transfection of mESCs with the pO4dERFP-IRESpuro-pA reporter construct resulted in weak red fluorescence in some of the cells in contrast to a stably integrated murine *Oct4*-GFP reporter construct, which showed high fluorescence in most of the cells. (Figure 5A-D). However, transfection with H₂O was negative (Figure 5E-H) and transfection with a control vector constitutively expressing dERFP yielded comparable low red fluorescence (data not shown).

***OCT4* and *NANOG* reporter expression in SCNT produced embryos**

Porcine fetal fibroblast cells containing the pO4-EGFP and pNG-dEGFP reporter constructs were used in both in-vitro and in-vivo cloning experiments. The in-vitro experiments included 5 and 4 SCNT experiments with PFFs containing the *OCT4* and *NANOG* reporter constructs, respectively

(Table 2). In addition, a single experiment using PFFs without constructs was performed (negative control). The experiments included reconstruction of 25-50 oocytes per construct per day and the rate of donor cell fusion and initial cleavage rates were ~75% and 55-95% respectively (Table 2). However, the blastocyst development rates were relatively low at approximately 15-18% (blastocyst developmental rates of 13 - 20% have previously been reported in the pig (Kurome et al., 2006)). In general, there were no apparent developmental differences between different groups of embryos containing the *OCT4* and *NANOG* reporter constructs. The same was true for the control embryos without constructs, although a slightly higher cleavage rate was observed. Green fluorescence was only observed in the cytoplasm of two 8-cell and one morula stage embryo containing the *OCT4* reporter construct and showed equal expression in all blastomeres (Table 2; Figure 6A), whereas, no fluorescence was observed at the blastocyst stage (Table 2; Figure 6E). Immunocytochemistry showed nuclear localized *OCT4* in blastomeres at the 8-cell to morula stage (Figure 6B) embryos, however, cytoplasmic localization of *OCT4* was most evident (Figure 6D). However, at the blastocyst stage, only nuclear localization of *OCT4* was observed (Figure 6 F-H). In contrast, green fluorescence was not observed in any of the examined embryo stages containing the *NANOG* reporter construct (Figure 6I) and staining with a *NANOG* antibody showed only cytoplasmic *NANOG* staining (Figure 6 J-L).

The in-vivo experiment included two SCNT experiments with reconstruction of around 50 oocytes from each of the *OCT4* and *NANOG* reporter constructs, which were transferred into two different recipient sows after 1-2 days of in-vitro culture (Table 3). Comparable fusion rates to the in-vitro experiment was observed with the *OCT4* and *NANOG* reporters, although a slightly higher cleavage rate at around 85% was observed. No Day 11-12 embryos were obtained following transfer of the pO4-EGFP SCNT embryos, which was presumably due to a lack of pregnancy (Table 3). However, several Day 11-12 embryos containing the *NANOG* reporter construct were isolated and staged (Vejlsted et al., 2006), including five ovoid and many filamentous embryos (Table 3). Unfortunately, green fluorescence was not detected in the embryos (Data not shown).

In an attempt to identify the possible reasons for the lack of green fluorescence, cDNA from pools of 5 in-vitro produced blastocysts containing either the *OCT4* or the *NANOG* reporter constructs as well as 5 Day 11-12 in-vivo produced embryos containing the *NANOG* reporter construct were analysed by RT-PCR. A positive control reaction with DNA purified from PFFs containing either the

OCT4 or the *NANOG* reporter constructs showed clear bands the gel (Figure 7A and B, lane 1). A faint band was also detected using cDNA of blastocysts containing the *OCT4* reporter construct as template (Figure 7A, lane 2), but not with cDNA of blastocysts containing the *NANOG* reporter construct (data not shown). In contrast, a clear band was obtained with cDNA of D11-12 embryos containing the *NANOG* reporter construct (Figure 7B, lane 2). Negative control reactions lacking RT enzyme or with H₂O as template showed no signals (Figure 7A and B, Lane 3 and 4).

Finally, staining with a GFP antibody was carried out on in-vitro produced 8-cell, morula and blastocyst stage embryos containing both reporter constructs and on D11-12 embryos containing the *NANOG* reporter construct, however, no specific fluorescence was observed (data not shown).

***OCT4* and *NANOG* reporter expression in SMGT produced embryos**

Green fluorescence was consistently observed in 1-cell to morula-stage embryos containing the *OCT4* reporter construct, but was only observed in individual blastomeres and thus presented a mosaic expression pattern (Figure 8A). Furthermore, the fluorescence seemed to correlate with OCT4 staining (Figure 8B-D). In contrast, green fluorescence was not observed in blastocysts containing the *OCT4* reporter construct. With the *NANOG* reporter construct the embryos showed green fluorescence from the 1-cell stage to the morula stage also presenting a mosaic expression pattern. Green fluorescence was furthermore observed in three blastocysts containing the *NANOG* reporter construct, which was localized exclusively in the ICM (Figure 8E). However, staining with a NANOG antibody was negative (Figure 8F-H).

Discussion

The expression pattern of porcine *OCT4* and *NANOG* has been subject to much attention and it has been argued that these transcription factors are not important for maintaining the pluripotent state in the pig (Keefer et al., 2007; Carlin et al., 2006; Kuijk et al., 2008; Blomberg et al., 2008). However, recent studies have shown a similar expression patterns of *OCT4* and *NANOG* compared to the human (Vejlsted et al., 2006; Hall et al., 2009). The aim of this study was to isolate the porcine *OCT4* and *NANOG* promoter regions and analyse them by alignments with the promoter regions of other mammals and by expression of fluorescent reporters in pluripotent cells and transgenic embryos.

The picture emerging from studies on regulation of *Oct4* and *Nanog* in the mouse and human is that these pluripotency markers are regulated by a combination of transcription factors as well as DNA methylation and chromatin remodelling. The binding sites identified in the porcine *OCT4* promoter region were, for the most part, highly conserved. The conserved sequences CCCTCCCCC and AGATGCATGACAAAG identified at approximately -2 kb in the porcine *OCT4* promoter correspond to the sites 2A and 2B, respectively, which makes up the DE of the murine promoter. Whereas site 2A has been shown to bind to an unknown transcription factor, possibly SP1/SP3, site 2B has been shown to bind a OCT4/SOX2 complex (Okumura-Nakanishi et al., 2005).

The conserved sequence TCCAGGCCCATTC AAGGGTTGAGCAC located at around -1 kb corresponds to the binding site 1B in the PE which has been shown to be involved in retinoic acid mediated downregulation during differentiation in the mouse (Okazawa et al., 1991). The PE is reported to be responsible for *OCT4* expression in the epiblast and in embryonal carcinoma (EC) cells (Okazawa et al., 1991). Interestingly, the site 1A, which forms the second half of the PE in the mouse, is almost completely missing in the pig. However, it was recently shown that this region is not conserved in other ungulates and primates and may not be important for regulation of *OCT4* (Kobolak et al., 2009).

The conserved sequence GGGGGCGGGGCCAGAGGTCAAGGCTA located around -50 bp, which corresponds to the SP1/SP3 transcription factor binding site in the PP, is believed to regulate initiation of transcription from TATA-less promoters, such as *Oct4* (Ovitt and Scholer, 1998). This sequence partly overlaps with a hormone responsive element, which can bind molecules induced by

retinoic acid and has been shown to be involved in downregulation of *Oct4* upon differentiation (Schoorlemmer et al., 1994; Minucci et al., 1996).

Finally, several sequences containing the consensus GGG(A/T)GGG or CCC(A/T)CCC have been identified in the porcine *OCT4* promoter, which are putative binding sites, however, so far their function remains unknown (Kobolak et al., 2009).

In the porcine *NANOG* promoter, several areas within the PP region showing high sequence identity with other mammals were identified. The sequence TAAAAAGTGGAAACAAGGTGGACCTGCAA located at approximately -250 was quite conserved between primates and ungulates but did not show significant sequence identity with a reported FOXD3 binding site, which has been shown to upregulate *Nanog* expression in the mouse (Pan and Thomson, 2007). Hence, it may represent an unknown transcription factor binding site in ungulates and primates.

The sequence GTCTG located around -175 bp was completely conserved in all the examined mammals and along with three AGAC sequences these sites are reported to act as SMAD binding sites in the human (Xu et al., 2008). In hESCs, the TGF β /Activin pathway has been shown to enhance the activity of *NANOG* through direct binding of SMADs. Since the AGAC sequences are quite conserved in the porcine *NANOG* promoter but shows more variation in the rodents, it could point to an active role of TGF β /ACTIVIN signaling in the pig as well.

The sequence, TTTGCATTACAATG located around -150 bp is reported to be an OCT4/SOX2 binding site necessary for the major promoter activity of *NANOG* (Kuroda et al., 2005). Furthermore, the sequences GGGGGTGTG and GGGGCGGGC located around -75 bp in the porcine promoter sequence corresponds to two SP1/SP3 transcription factor binding sites (Wu and Yao, 2006). In the mouse and human, it was recently shown that KLF4 binds to these sites and directly interacts with OCT4/SOX2 in regulating the expression of *NANOG* (Chan et al., 2009; Wei et al., 2009). The observation that binding sites of OCT4, SOX2 and KLF4 are conserved in all the mammals, including the pig is particularly interesting, as these transcription factors are reported to be crucial and sufficient for reprogramming of fibroblasts into iPSCs (Nakagawa et al., 2008).

A TATAAA site was furthermore identified at -30 bp, which is usually involved in transcription by RNA polymerase II. It has been reported that TATA less promoters allows for a fast transcription switch-off which is important in differentiating cells (Ovitt and Scholer, 1998). The reason why a TATA site was observed in the *NANOG* promoter of ungulates and primates, but not in rodents is unknown, however, it could be speculated that other sites are involved in downregulation of *NANOG* in rodents.

In the mouse, negatively regulating cis elements are furthermore reported to bind in the -5 kb promoter region (Chan et al., 2009), however, so far the corresponding area in the porcine *NANOG* promoter has not been sequenced. In this region, Smad1 has been shown to bind to the sequences GCCGCGCCA, GCCGCACC and GCCACGGC and STAT3 and Brachyury to the sequences TTCCTAGAA and GGGACACACCTAGGGTTCCC, respectively. In concert, these transcription factors are reported to block bone morphogenic protein (BMP) induced differentiation in mESCs (Suzuki et al., 2006). Furthermore, it has been suggested that leukemia inhibitory factor (LIF) signaling through STAT3 can upregulate *Nanog* (Pan and Thomson, 2007), which may explain why overexpression of *Nanog* can maintain mESCs in their undifferentiated state without supplementation of LIF (Chambers et al., 2003).

As stable ESC-lines expressing pluripotency markers such as *OCT4* and *NANOG* currently do not exist in the pig, another type of pluripotent cell was used to analyse the porcine pluripotency reporter constructs. Since the murine *Oct4* promoter has been shown to be functional in porcine embryos (Kirchhof et al., 2000), it was hypothesized that the porcine *OCT4* reporter construct might also be functional in mESCs. However, only weak fluorescence was observed in mESCs transfected with the *OCT4*-dERFP construct in contrast to a reporter construct of murine *Oct4*, which displayed more widespread green fluorescence. The observation that constitutively expressed dERFP was also expressed at a low level in mESCs may point to inefficiency in translation of the fluorophore as fluorophores are known to be expressed differently in various types of cells. In one study, dERFP was found to be expressed at different levels in different tissues, with eg. low level expression in mouse lung and liver but with high expression in mouse muscle (Bell et al., 2007). A human *OCT4* reporter construct which was found to be expressed in mouse fetal fibroblast cells has furthermore demonstrated, that the mouse can not always be used to study tissue-specific changes in other species (Cha et al., 2008). In contrast, the recently generated porcine iPSCs could serve as a more reli-

able cell type for in-vitro analysis of the pluripotency reporter constructs, since stable expression of *OCT4* and *NANOG* at a comparable level to hESCs have been reported in these cells (Wu et al., 2009).

Examination of *OCT4* and *NANOG* reporter constructs in cloned porcine preimplantation embryos yielded only green fluorescence in three 8-cell to morula stage embryos carrying the porcine *OCT4* reporter construct, whereas, fluorescence was not observed in embryos containing the *NANOG* reporter construct. The most obvious explanation for this may be a different expression profile of *OCT4* and *NANOG* in the pig, compared to the mouse and human. Accordingly, staining with a NANOG antibody did not show nuclear localized NANOG in any of the stages, indicating that the blastocyst stage is perhaps not optimal for the study of pluripotency markers in the pig. However, as staining with an OCT4 antibody did show nuclear localized OCT4 at the blastocyst stage, one would expect fluorescence in all the cells of the blastocyst carrying the *OCT4* reporter construct.

A more plausible explanation is that silencing of the exogenous pluripotency reporter constructs occurs, as it has previously been shown that reporter constructs are subject to epigenetic silencing in hESCs (Liew et al., 2007). In a recent study, a human *OCT4* reporter construct containing a 4 kb upstream sequence was completely silenced 72 hours after nucleofection and G418 selection in hESCs and EC cells, which was primarily attributed to in-vitro methylation (Stewart et al., 2008). In contrast, the CMV promoter driving G418 was not silenced possibly due to the selection pressure exerted on this promoter. It is possible that a similar mechanism is responsible for silencing of the porcine *OCT4* and *NANOG* reporter constructs in nucleofected PFFs during selection in G418. It could be interesting to apply antibiotic selection to mESCs transfected with pO4dERFP-IRESpuromycin to examine if *OCT4* was capable of driving puromycin resistance in these cells.

A final possibility is that post-transcriptional modifications could be responsible for the lack of reporter expression. RT-PCR showed GFP expression in blastocysts containing the *OCT4* reporter construct, as well as in Day 11-12 embryos containing the *NANOG* reporter construct, whereas, staining with a GFP antibody did not yield any specific staining. It is likely that splicing, which normally prevents the expression of intron-lacking pseudogenes, inhibits the conversion of reporter RNA to messenger RNA due to the lack of introns. This hypothesis is supported by the fact that a

mouse reporter construct, containing the entire Oct4 gene including introns, could be expressed in porcine embryos (van Eijk et al., 1999).

Using SMGT, fluorescence of the *OCT4* reporter construct was consistently observed from around the 1-cell stage to the early morula stage embryo, after which the fluorescence was lost. This observation fits with a gradual silencing of the *OCT4* reporter construct during in-vitro culture. In contrast, with this method the *NANOG* reporter construct was found to be localized exclusively in the ICM of blastocysts, but since *NANOG* was not detected by immunocytochemistry it seems that only *NANOG* transcripts are present at this stage. In general, with SMGT the location and number of transgene integration sites are reported to fluctuate (Habermann et al., 2007). This could explain the observed mosaic expression profile of the *OCT4* and *NANOG* reporter constructs in SMGT embryos in contrast to SCNT embryos in which a ubiquitous green fluorescence was observed with the *OCT4* reporter. Furthermore, a higher number of reporter construct integrations could explain why expression of the *NANOG* reporter construct was only detected by SMGT and not by SCNT. However, as the SMGT technique has not been completely optimized yet, further studies are required to verify correct expression of the pluripotency reporters, including fluorescent in situ hybridization to analyse integration of transgenes.

Several steps could be taken to ensure correct expression of the reporter constructs in porcine cells and tissues. Inclusion of additional enhancer elements, especially in the 3' region has been shown to reduce silencing in hESCs and EC cells (Stewart et al., 2008). However, in the case of *OCT4*, all the known enhancers were already included. Liew and colleagues have shown that addition of a polyoma virus mutant enhancer called PyF101 retains reporter driven EGFP expression in hESCs and EC cells (Liew et al., 2007). A similar approach may be applied to the porcine pluripotency reporter constructs. However, the most reliable way to overcome silencing as well as incorrect splicing is by use of homologous recombination, which could either be carried out by BAC recombineering, or by directly inserting a reporter protein in front of *OCT4* and *NANOG* by use of targeting vectors. The latter, however, could have serious implications in the maintenance of pluripotency, as has previously been shown in mESCs (Niwa et al., 2000; Chambers et al., 2003).

Conclusion

This study provides the first comprehensive analysis of the porcine *OCT4* and *NANOG* promoter regions. Detailed sequence analysis showed that the porcine *OCT4* and *NANOG* promoter regions contain most of the transcription factor binding sites reported to be involved in maintaining the pluripotent state in the mouse and human, which points to an important role of *OCT4* and *NANOG* in the pig as well. Interestingly, most of the transcription factor binding sites showed the highest sequence identity with primates compared to rodents. For the generation of a pluripotent reporter pig, the *OCT4* and *NANOG* reporter constructs need further verification and testing in pluripotent cells or transgenic embryos, expressing stable levels of porcine *OCT4* and *NANOG*.

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Figure legends

Figure 1. Alignment of *OCT4* promoter region. *OCT4* promoter region in seven species were compared. A 7.8kb alignment containing the 1st exon and the upstream regulatory sequence was analysed. The distal enhancer (DE), proximal enhancer (PE) and proximal promoter (PP) are shown with transcription factor binding sites indicated. Nucleotides which are conserved among the seven species are bolded and shown in a blue background. Repetitive elements which have been identified by BLAST search are indicated in dark green. The 5'-untranslated region is shown in light red, whereas, the open reading frame is shown in dark red. The regulatory region used for the *OCT4* reporter construct in the present study is shown above the alignment scheme.

Figure 2. Alignment of *NANOG* promoter region. *NANOG* promoter region in six species were compared. A 3.9kb alignment containing the 1st exon and the upstream regulatory sequence was analysed. The proximal promoter (PP) is shown with transcription factor binding sites indicated. Nucleotides which are conserved among the seven species are bolded and shown in a blue background. Repetitive elements which have been identified by BLAST search are indicated in dark blue. The 5'-untranslated region is shown in light orange, whereas, the open reading frame is shown in dark orange. The regulatory region used for the *NANOG* reporter construct in the present study is shown above the alignment scheme.

Figure 3. PCR analysis of BACs. Characterization of BACs by PCR. (A) PCR with primers of porcine *OCT4*. Lane 1: Pig chromosomal DNA, lane 2: BACRP44-400E7, lane 3: BAC242-102G9, lane 4: BAC242-83M14. (B) PCR with primers of porcine *NANOG*. Lane 1: Pig chromosomal DNA, lane 2: BAC242-335, lane 3: BAC242-172H17. All panels show 1% agarose gel stained with ethidium bromide.

Figure 4. Overview of the *OCT4* and *NANOG* reporter constructs. Schematic representation of the reporter constructs used in the present experiment. (A) The red fluorescent *OCT4* reporter construct pO4dERFP-IRESpuro-pA showing a constitutively expressed G418 gene (orange), the 6.6 kb *OCT4* promoter region (blue), a destabilized, enhanced red fluorescent protein (red) and an IRE-SpuroPA cassette (grey). (B) The green fluorescent *OCT* reporter construct pO4-EGFP showing a 6.6 kb *OCT4* promoter region (blue), an enhanced green fluorescent protein (green) and a constitu-

tively expressed neomycin resistance gene flanked by loxP sites (orange). (C) A colony blotting membrane hybridized with a *NANOG* DNA probe. (D) Restriction pattern of clones containing *NANOG* promoter region using different restriction enzymes. (E) The green fluorescent *NANOG* reporter construct pNG-dEGFP showing a 2.2 kb *NANOG* promoter region (purple), a destabilized, enhanced green fluorescent protein (green) and a constitutively expressed neomycin resistance gene flanked by loxP sites (orange).

Figure 5: mESC containing a red fluorescent *OCT4* reporter construct. mESCs containing a murine green fluorescent *Oct4* reporter construct transfected with pO4dERFP-IRESpuro-pA. (A-D) mESCs transfected with a porcine red fluorescent *OCT4* reporter construct. (E-H) mESCs transfected with H₂O (negative control). (A and E) Bright field image om mESC colonies. (B and F) Green fluorescence of murine *Oct4* reporter construct (C and G) Red fluorescence of porcine *OCT4* reporter construct (D and H) Merge. Scale bars represent 0.05 mm.

Figure 6. RT-PCR analysis of SCNT embryos containing green fluorescent *OCT4* and *NANOG* reporter constructs. Characterization of SCNT embryos by RT-PCR with primers of GFP. (A) Lane 1: DNA from PFFs containing pO4-dEGFP, lane 2: cDNA from 5 pooled blastocysts containing pO4-EGFP, lane 3: -RT enzyme, lane 4: H₂O (negative control). (B) Lane 1: DNA from PFFs containing pNG-dEGFP, lane 2: cDNA from 5 pooled D11-12 embryos (epiblasts) containing pNG-EGFP, Lane 3: -RT enzyme, Lane 4: H₂O (negative control). All panels show 3% agarose gel stained with ethidium bromide.

Figure 7. SCNT embryos containing green fluorescent *OCT4* and *NANOG* reporter constructs. Fluorescence of SCNT embryos containing pO4-EGFP or pNG-dEGFP (A-D) Morula stage embryo containing a green fluorescent *OCT4* reporter construct stained with a red fluorescent *OCT4* antibody. (E-H) Blastocyst stage embryo containing a green fluorescent *OCT4* reporter construct and stained with a *OCT4* antibody. (I-L) Blastocyst stage embryo containing a green fluorescent *NANOG* reporter construct stained with a red fluorescent *NANOG* antibody. (A, E and I) Green fluorescence (reporter construct). (B, F and J) Red fluorescence (antibody staining). (C, G and K) DAPI staining of nuclei. (D, H and L) Merge. Scale bars represent 0.05 mm.

Figure 8. SMGT embryos containing green fluorescent *OCT4* and *NANOG* reporter constructs. Fluorescence of SMGT embryos containing pO4-EGFP or pNG-dEGFP (A-D) 4-cell stage embryo containing a green fluorescent *OCT4* reporter construct stained with a red fluorescent OCT4 antibody. (E-H) Blastocyst stage embryo containing a green fluorescent *NANOG* reporter construct stained with a red fluorescent NANOG antibody. (A and E) Green fluorescence (reporter construct). (B and F) Red fluorescence (antibody staining). (C and G) DAPI staining of nuclei. (D and H) Merge. Scale bars represent 0.05 mm.

Table 1. Primers used for generation of reporter constructs, PCR and RT-PCR.

Primer name	Sequence (5'-3')	Annealing emp.	Product size
5armEHf	ctgaGAATTCagtcGTTAACtttgctgtacacctgaaac	75°C	436 bp
5armNr	acctgtGCTAGCatgcactgctcatgcca	78°C	
3armNf	aggtagGCTAGCttgaggagacagtgccag	72°C	348 bp
3armBr	gtctgcAGATCTccaaggggactgctc	72°C	
XadN53	CTAGTcgaggacttcctgaGTGCA	64°C	25 bp
XadN35	CtcaaggaagtggtcgA	48°C	
NG6f	gctagattggggtggttagc	57°C	195 bp
NG200r	cactcatgttgagttgaagaag	52°C	
pO4RegF	gttaaATTAAATctttgctgtacacctgaaac	64°C	6594 bp
pO4RegR	gtggaACCGGTtctccaaggggactgctc	78°C	
pOct4Fw	agggttcagccaaacgacc	60°C	898 / 268 bp
pOct4Rv	gccagaaggcacaacgatca	63°C	
pNanogF1	cgaatgaaatgtaagaggt	47°C	300 / 160 bp
pNanogR1	gtggggtaatcagagctgg	55°C	
GFP_F2	actacagctccgtggtggac	57°C	119 bp
GFP_R2	ggtgttctgtgatcctct	57°C	

Table 2. In-vitro SCNT experiment.

Reporter	%Fusion	%Cleavage	%Fluorescent embryos	%Blastocysts	%Fluorescent blastocysts
pO4-EGFP	73 (141/192)	65 (91/141)	3 (3/91)	15 (16/141)	0 (0/16)
pNG-dEGFP	77 (113/147)	55 (62/113)	0 (0/113)	18 (16/113)	0 (0/16)
Neg. control	76 (22/29)	95 (21/22)	0 (0/22)	18 (4/22)	0 (0/4)

Table 3. In-vivo SCNT experiment.

Reporter	%Fusion	%Cleavage (Day 1)	%Embryos transferred	Pregnancy	%Fluorescent embryos
pO4-GFP	78 (108/138)	84 (36/43)	91 (98/108)	-	
pNG-GFP	81 (112/139)	83 (48/58)	90 (101/112)	+	0 (0/101)

Figure 1.



Figure 2.

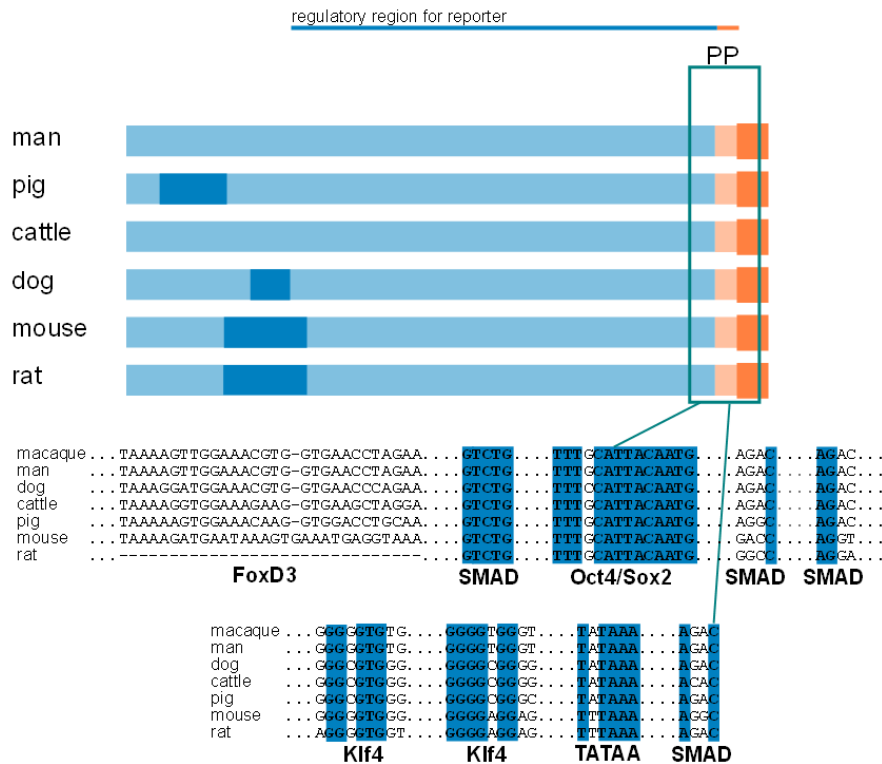


Figure 3.

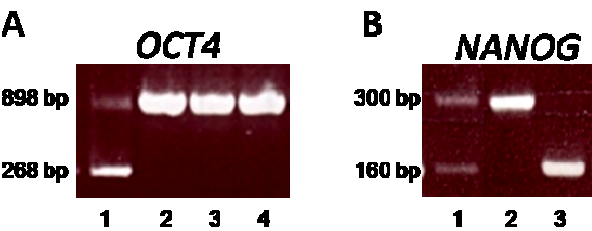


Figure 4.

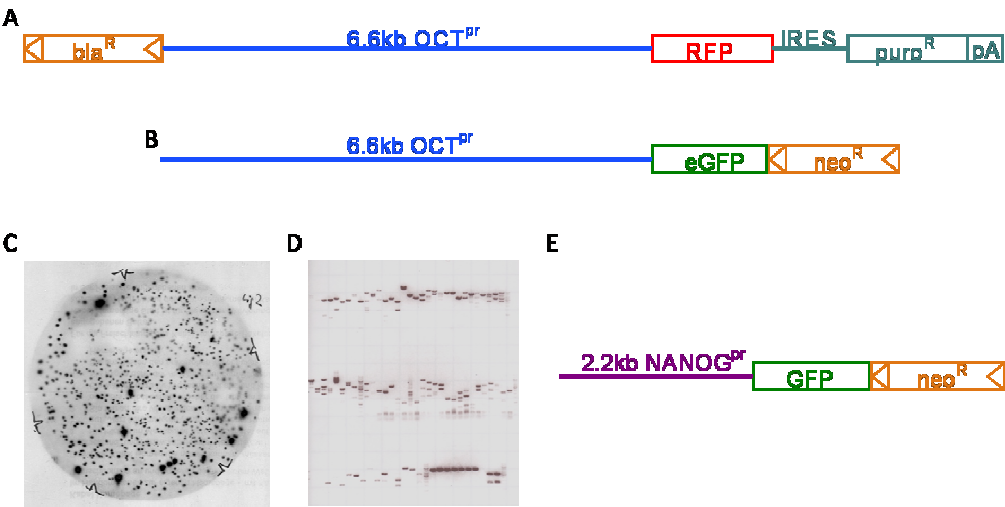


Figure 5:

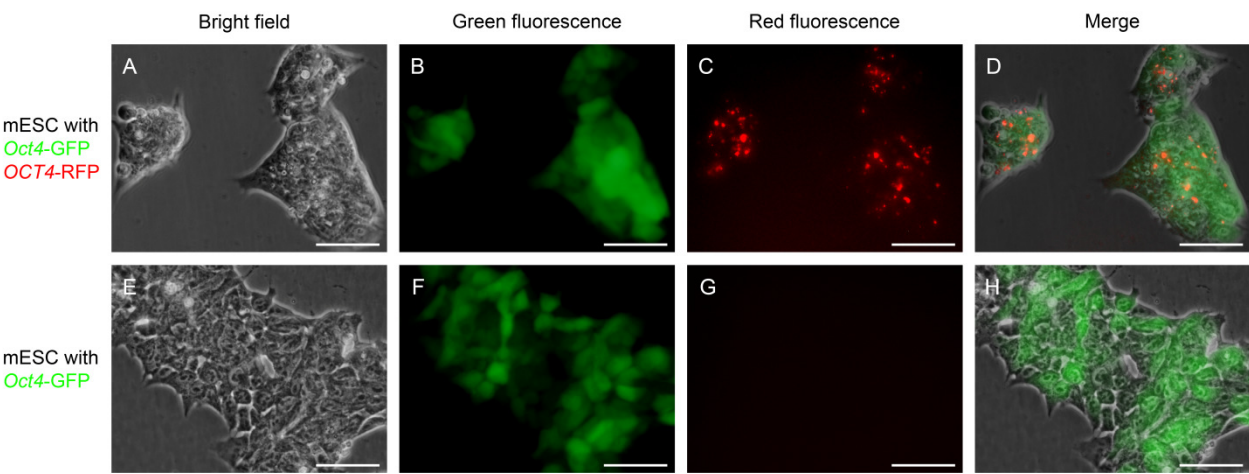


Figure 6.

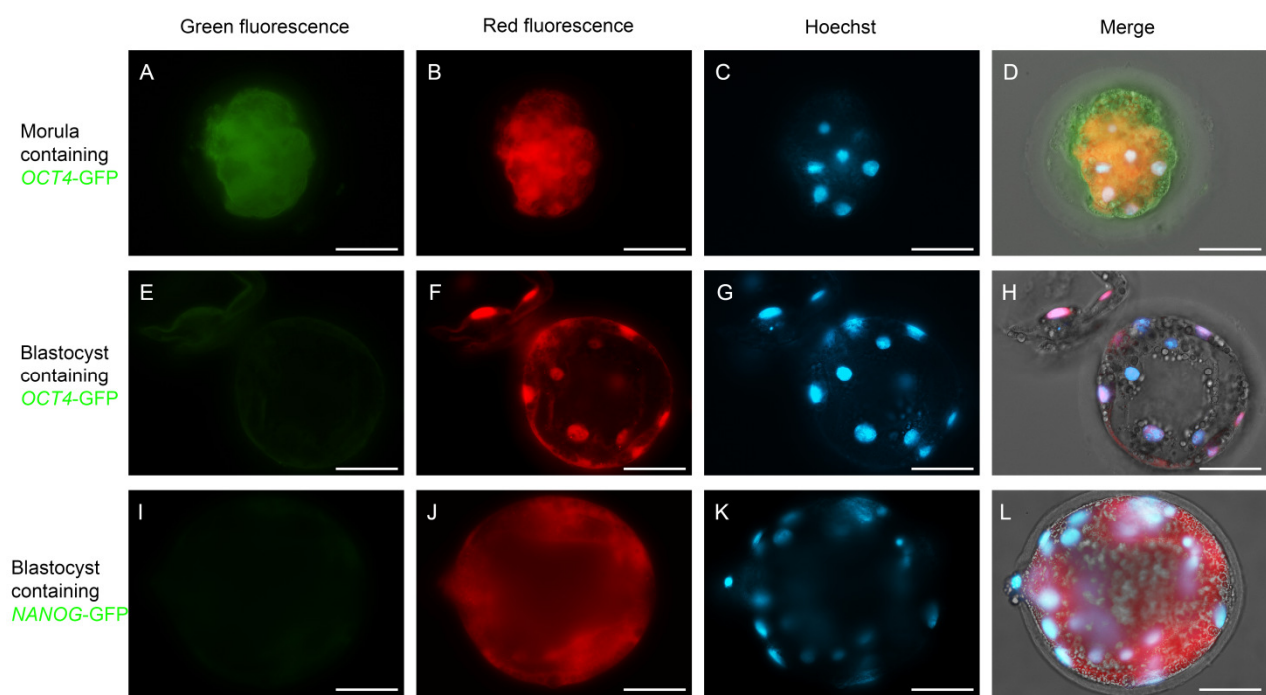


Figure 7.

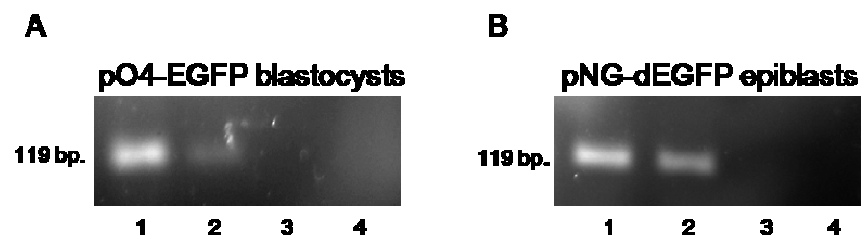
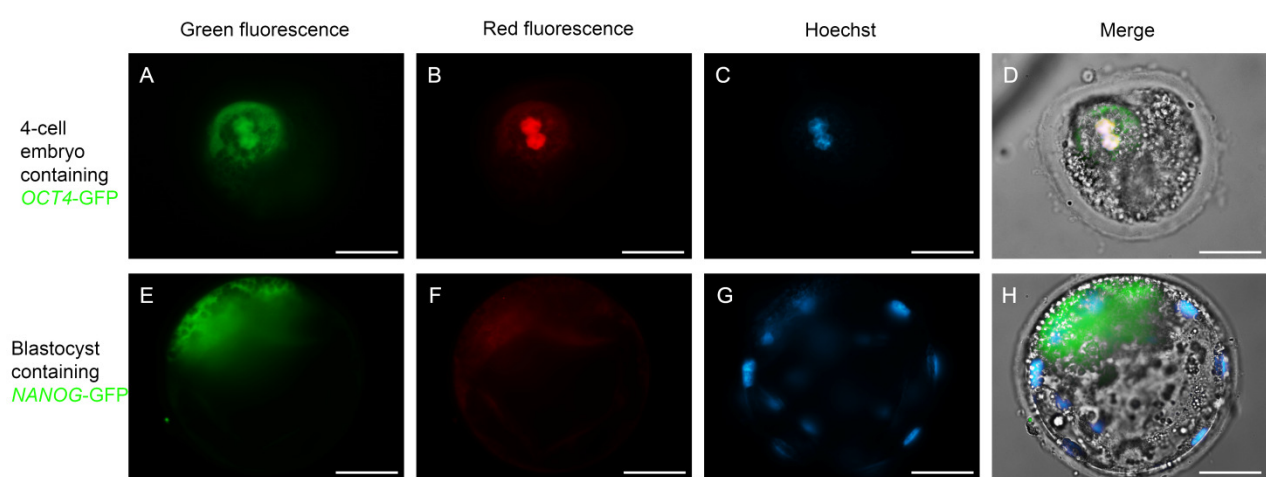


Figure 8.



7.3. Manuscript III. Directed differentiation of porcine epiblast-derived neural progenitor cells to mature neurons and glia

Title

Directed differentiation of porcine epiblast-derived neural progenitor cells to mature neurons and glia

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Key words: Epiblast, neural progenitor cells, differentiation, neurons, glia.

Running Title: Porcine epiblast-derived neural progenitor cells into mature neurons and glia

Abstract

Neural progenitor cells (NPCs), derived from embryonic stem cells (ESCs) are capable of self-renewal and differentiation into neural and glial lineages, making them promising candidates for cell-based therapy of neurodegenerative diseases. However, safety aspects need to be determined through transplantation into relevant animals such as the pig. The aim of this study was to derive NPCs from porcine epiblast cells and evaluate their differentiation potential. Epiblasts were isolated from embryos 9 days post insemination and cultured on mouse embryonic feeder cells in ESC medium. Outgrowth colonies were passaged to MS5 stromal cells where neural rosettes formed. When rosette structures were isolated and cultured in Matrigel-coated dishes in the presence of FGF and EGF, a homogeneous population of NPCs outgrew which has been maintained for more than 15 passages. Comparative real-time PCR and immunocytochemistry showed expression of the makers *KI67*, *SOX2*, *NESTIN*, *VIMENTIN*, and *PAX6* in 56%, 100%, 96%, 100% and 99% of the cells, respectively. The same markers were also identified in the lateral ventricles of the fetal porcine brain, a location known to harbour NPCs. NPCs were subjected to differentiation using five different growth factor combinations including (1) no growth factors (control), (2) RA and SHH (motoneurons), (3) FGF8 and SHH (dopaminergic midbrain neurons), (4) PDGF (oligodendrocytes), and (5) RA and LIF (astrocytes). *TUJ1*, a marker of immature neurons, was significantly upregulated in protocol 2 (58% positive cells) as were markers of mature neurons such as *NF* and *TH*. In contrast, the oligodendrocyte marker *MBP* was significantly upregulated in protocol 3 (63% positive cells), whereas the astrocyte marker, *GFAP*, was significantly upregulated in protocol 1, 2, 3, and 4 (33%, 25%, 43% and 22%, respectively). The present study provides the first evidence of a porcine epiblast-derived NPC-line, capable of differentiating into mature neurons and glia, which brings the pig one step closer as a model of human NPC therapy.

Introduction

Neural progenitor cells (NPCs) are multipotent and can form neurons, astrocytes and oligodendrocytes induced by either, instructive, paracrine cues, or by selective survival mechanisms (Mehler & Kessler 1999). NPCs can be isolated directly from the ventricular zone (VZ) from the developing fetus and subventricular zone (SVZ) from the adult (Mehler & Kessler 1999), but they can also be differentiated from embryonic stem cells (ESC). Their multipotency and ability to self-renew *in vitro* render these cells interesting candidates for cell-based therapy of neurodegenerative diseases such as Alzheimer's and Parkinson's disease. Rodents, such as the mouse and the rat, are well-established models for neurotransplantation studies. Such research has shown that transplantation of NPCs can improve cognition in a mouse model of Alzheimer's disease (Blurton-Jones *et al.* 2009), induce remyelination in a mouse model of multiple sclerosis (Pluchino *et al.* 2003), and improve locomotion and respiration in a rat model of Amyotrophic Lateral Sclerosis (Lepore *et al.* 2008). Furthermore, several groups have shown that ESC-derived dopaminergic neurons can induce restitution of motor function in a rat model of Parkinson's disease (Geeta *et al.* 2008; Roy *et al.* 2006; Yang *et al.* 2008). In contrast to these promising results, a recent treatment of a human Ataxia Telangiectasia patient with NPCs derived from human fetuses resulted in development of a malignant brain tumour (Amariglio *et al.* 2009). This unfortunate outcome pinpoints that more detailed pre-clinical studies are required to minimize the risks. As new studies outline considerable differences in the brain between mice and humans, including differences in astrocyte activity (Oberheim *et al.* 2009) and in the aging processes (Oh *et al.* 2009), safety studies of NPC-transplantations in other animal models than rodents is of vital importance.

Large animal models may be of utmost importance in the field of translational neuroscience, as a tool for testing safety and potentials of NPC-treatment. The pig is an excellent candidate, as it resembles man in both size, anatomy and physiology (Lind *et al.* 2007); all of which are important aspects when studying diseases affecting a complex organ, like the brain. The minipig is particularly suitable as a model of Parkinson's disease due to the similar anatomy of the substantia nigra (Nielsen *et al.* 2009) and development of a stable Parkinsonian-like syndrome in response to the chemical MPTP (Bjarkam *et al.* 2008). Recent advances in somatic cell nuclear transfer has resulted in the generation of a transgenic minipig model of human Alzheimer's disease (Kragh *et al.* 2009).

As an extension, transgenic in-vitro disease models could be established by derivation of porcine NPC (pNPC) lines from transgene embryos allowing in-vitro drug screening.

In the fetal human brain, NPCs isolated from the cerebral cortical VZ are reported to express the transcription factors *SOX1* and *SOX2*, the intermediate filament *NESTIN*, as well as the RNA binding protein *MUSASHI* (Shin *et al.* 2007). In the adult brain, NPCs are located in the subventricular zone (SVZ) of the lateral ventricular wall and in the subgranular zone (SGZ) of the hippocampal dentate gyrus (Ma *et al.* 2009). These cells have unique molecular profiles, which are likely due to the microenvironment of the varying regions of the brain where they reside. Adult, actively dividing NPCs isolated from the SVZ, express *SOX2* and *NESTIN*, in addition to the homeobox transcription factor *paired box gene 6 (PAX6)* (Ma *et al.* 2009). In contrast, adult quiescent NPCs, corresponding to radial glial-like cells, express *NESTIN*, *glial fibrillary acidic protein (GFAP)* and *stage specific embryonic antigen 1 (SSEA1)* (Ma *et al.* 2009). Although the fundamental NPC-markers seems to be shared between fetal and actively dividing adult NPCs, the vast majority of genes are differentially expressed, indicating a fundamental difference in the way these cells maintain their neuroprogenitor state (Maisel *et al.* 2007).

NPCs have also been established in-vitro from pluripotent cells, as e.g. ESC, which is an attractive alternative to brain-derived NPCs due to their potentially, unlimited supply. NPCs were first derived from murine ESCs (mNPCs) in 1996, and these cells proved capable of differentiating into both neurons and glial cell types (Okabe *et al.* 1996). Later, NPCs were also derived from human ESCs (hNPCs) (Reubinoff *et al.* 2000) and their potential to differentiate into neurons and glial cells was verified (Reubinoff *et al.* 2001). ESC-derived hNPCs share many markers with their fetal counterparts such as expression of *SOX1*, *SOX2*, *NESTIN* and *MUSASHI*. However, differences in expression are also evident, especially within key differentiation pathways such as *leukemia inhibitory factor (LIF)*, *basic fibroblast growth factor (bFGF)* and *Wnt* signaling (Shin *et al.* 2007).

To date, several methods for derivation of NPCs from hESC have been reported (Schwartz *et al.* 2008). Although the neural differentiation pathway is reported to function as a default mechanism in hESC, most methods relies on neural induction either by formation of embryoid bodies (EBs) or by co-culture with different types of mouse stromal cells (Schwartz *et al.* 2008). Whereas EBs can directly give rise to hNPCs, neural rosettes, which are morphologically reminiscent of the develop-

ing neural tube (Lazzari *et al.* 2006) give rise to hNPCs in the co-culture method. Human NPCs are furthermore capable of long term culture in an undifferentiated state when cultured in the presence of bFGF and epidermal growth factor (EGF) (Andersen *et al.* 2009).

NPC carry the potential to differentiate into three different lineages: neurons, astrocytes and oligodendrocytes (Erceg *et al.* 2009). When growth factors are withdrawn from hNPCs, a mixed population of neurons and glial cells will spontaneously form (Joannides *et al.* 2007). In contrast, treatment of NPCs with various growth factors can promote differentiation into more specialized cell types. Retinoic acid (RA), which plays an important role in the development and maintenance of the nervous system (Maden 2007), has been shown to promote formation of motoneurons from hESC-derived NPC (Lee *et al.* 2007; Li *et al.* 2005). Other factors such as SHH, which is crucial for patterning of the ventral neural tube (Patten & Placzek 2000), and FGF8, which is involved in organizing the embryonic mid- and hindbrain (Crossley *et al.* 1996), have been shown to promote the formation of dopaminergic neurons (Park *et al.* 2005; Perrier *et al.* 2004; Roy *et al.* 2006; Yan *et al.* 2005). In addition, platelet derived growth factor (PDGF) has been shown to stimulate oligodendrocyte formation (Hu *et al.* 2008; Kang *et al.* 2007). In contrast, pathways involved in astrocyte differentiation remains poorly understood and these cells are mostly generated spontaneously (Trownson 2006). However, it was recently shown that astrocyte formation can be induced from mNPC by addition of RA and LIF (Asano *et al.* 2009).

At present, the possibility of establishing porcine NPCs (pNPCs) from ESCs is hampered by the fact that pluripotent ESC cultures cannot be maintained long-term in this species (Vackova *et al.* 2007). However, research has indicated that long term culture of porcine epiblast cells results in spontaneous generation of immature astrocytes and neurons (Talbot *et al.* 2002). Thus, the neural pathway may function as the default mechanism in this species. Lazzari and co-workers were the first to derive neuroectoderm directly from the inner cell mass (ICM) of bovine preimplantation embryos, and the resulting rosettes gave rise to a population of neural crest progenitor cells which could be maintained in-vitro for more than 112 days. When growth factors were withdrawn these cells were able to differentiate into mature neuronal and glial subtypes in addition to chondrocytes and smooth muscle cells (Lazzari *et al.* 2006). Recently, Du and co-workers have shown that porcine ICM cells can be differentiated into pNPC and maintained for up to 2 months in-vitro (Du *et al.* 2009). However, the differentiation potential of these cells was limited to glial cells, including

astrocytes and oligodendrocytes. Recent reports on generation of porcine induced pluripotent stem cells (iPSC) may open up exciting possibilities of using iPSC derived pNPCs as a model of isogenic NPC-therapy (Esteban *et al.* 2009; Ezashi *et al.* 2009; Wu *et al.* 2009).

To realize the potential of using the pig as a large animal model of NPC-treatment, the need for a stable embryo derived pNPC-line with the capacity to differentiate into mature neurons and glial cells is apparent. The aim of the present study was (1) to isolate porcine pNPCs from epiblast cells and characterize these by means of established hNPC markers, and (2) to evaluate the potentials of the pNPCs for long-term culture and differentiation into mature neurons and glia.

Materials and Methods

Unless otherwise stated materials were purchased from Invitrogen, Carlsbad, CA.

Isolation and culture of epiblast cells

Uteri from two Danish sows (Landrace x Yorkshire crosses) were artificially inseminated over 2 days with semen from Duroc boars and collected at a local abattoir 9 days post insemination (Day 9). Each uterine horn was flushed with 150 ml embryo transfer solution (LIFE Pharmacy, KU, Frederiksberg, Denmark) containing 0.1% FBS and collected via a flushing catheter. Embryos were subsequently isolated in DMEM containing HEPES (Sigma-aldrich, St. Louis, MO) and 10% FCS by stereo-microscopy.

Epiblasts from Day 9 expanded hatched blastocysts (Vejlsted *et al.* 2006) were mechanically isolated from the surrounding trophoctoderm and hypoblast, using insulin needles. The isolated epiblasts were cultured as outgrowth colonies (OCs) in dishes containing $2 \times 10^4/\text{cm}^2$ mouse embryonic fibroblast (MEF) feeder cells (DSMZ, Braunschweig, Germany) in ESC medium consisting of knockout DMEM (Sigma-aldrich), 10% knockout serum replacement (KSR), 5% FCS, 1% penicillin/streptomycin (Sigma-aldrich), 1% non-essential amino acids (Sigma-aldrich), 0.2% beta-mercaptoethanol (Sigma-aldrich), 20 ng/ml human basic fibroblast growth factor (bFGF) and 20 ng/ml human Activin A (R&D systems, Minneapolis, MN) and cultured at 38°C in 20% O₂, 5% CO₂ in N₂.

Derivation and culture of pNPCs

An overview of the derivation and differentiation of pNPCs is presented in Figure 1. Mouse stromal cells (MS5; DSMZ) were cultured for one day in gelatin-coated dishes in a medium consisting of alpha-MEM medium (Sigma-aldrich) containing 10% FBS. On Day 5 following epiblasts-isolation, ESC-like areas of OCs were cut into 4-12 small pieces by use of insulin needles and co-cultured with $2 \times 10^4/\text{cm}^2$ MS5 cells in serum replacement medium (SR medium) containing DMEM, 15% KSR and 2 mM L-glutamine (Perrier *et al.* 2004). The cells were cultured at 38°C in 20% O₂, 5% CO₂ in N₂ and medium was replaced every 2-3 days without passage. Rosettes, which typically appeared after 12-17 days co-culture, were isolated by insulin needles, cut into small pieces and transferred to Matrigel-coated dishes (BD Biosciences, Franklin Lakes, NJ) in medium containing DMEM/F12, 1xB-27 supplement, 1xN2 supplement, 20 ng/ml EGF and 20 ng/ml bFGF and cul-

tured at 38°C in 20% O₂, 5% CO₂ in N₂ with medium change every 2-3 days. After 8 days, appearing NPCs were disaggregated in 1% Trypsin/EDTA and split into new Matrigel-coated dishes (BD Biosciences) at a ratio of 1:5 with subsequent passage every 3-4 days. For analysis of cell doublings, individual NPCs were counted at each passage using a haemocytometer and total doublings were calculated. Cells from passage 1 (primary culture of rosettes), 3 and 15 were sampled for comparative real-time PCR analysis and from passage 4 and 16 for immunocytochemical analysis.

Differentiation of pNPCs into neurons and glia.

To evaluate the differentiation potential of pNPCs, five alternate differentiation protocols were performed. pNPCs at passage 4 were disaggregated into single cells and seeded at a density of $1 \times 10^5/\text{cm}^2$ in Matrigel-coated dishes (BD Biosciences) and dishes containing Matrigel-coated glass coverslips (ThermoFisher Scientific, Waltham, MA). The cells were cultured for a total of three weeks in N2 medium consisting of DMEM/F12 and 1xN2 supplement in the following conditions: Protocol 1 (*mixed neurons and glia*); N2 medium without growth factors (Joannides *et al.* 2007). Protocol 2 (*motoneurons*); two weeks culture in N2 medium containing 1 μM all-*trans*-retinoic acid (RA; Sigma-aldrich), 200 ng/ml recombinant murine sonic hedgehog (SHH), 20 ng/ml human recombinant brain-derived neurotrophic factor (BDNF, Prospec, Rehovot, Israel), 0.2mM ascorbic acid (AA; Sigma-aldrich) followed by one week maturation in 20 ng/ml human recombinant glial cell line-derived neurotrophic factor (GDNF, Prospec, Rehovot, Israel), 20 ng/ml BDNF (Prospec) and 0.2mM AA (Sigma-aldrich) (Lee *et al.* 2007). Protocol 3 (*Dopaminergic midbrain neurons*); two weeks culture in 200 ng/ml SHH, 100 ng/ml human recombinant fibroblast growth factor 8 (FGF8), 20 ng/ml BDNF (Prospec) and 0.2mM AA (Sigma-aldrich) followed by one week maturation in 20 ng/ml GDNF (Prospec) and 20 ng/ml BDNF (Prospec) and 0.2mM AA (Sigma-aldrich) (Perrier *et al.* 2004). Protocol 4 (*Oligodendrocytes*); 20 ng/ml human recombinant platelet-derived growth factor-AB (PDGF; Sigma-aldrich) (Hu *et al.* 2008) and Protocol 5 (*Astrocytes*); 1 μM RA and 20 ng/ml recombinant murine leukemia inhibitory factor (LIF; Chemicon, Hessen, Germany) (Asano *et al.* 2009). Differentiation was performed at 38°C in 5% O₂, 5% CO₂ in N₂, with half of the media changed every third day. Only cells in protocol 3 and 4 were passaged 1:2 on Day 9 due to continued proliferation. At the conclusion of the experiment, three samples were obtained from each protocol for comparative real-time PCR and two samples were obtained for immunocytochemistry.

RNA purification and Reverse Transcription

pNPCs and differentiated cells were trypsinized into single cells, placed in lysis buffer (Qiagen, Chatsworth, CA), snap-frozen in liquid nitrogen and stored at -80°C. As positive control tissue, porcine brains from three fetuses, isolated from the uterus of a Danish sow (Landrace x Yorkshire crosses) 42 days post insemination (Day 42) (Vejlsted *et al.* 2006), were isolated from the skull and minced using a razorblade. As negative control tissue, MS5 cells were cultured as described above. Both were placed into lysis buffer, frozen in liquid nitrogen and stored at -80°C. Total RNA was purified with RNeasy mini or micro kit (Qiagen) and the RNA content and purity was measured on a Nanodrop 1000 spectrophotometer (ThermoFisher Scientific). Reverse transcription was performed using RevertAid First strand cDNA synthesis kit (Fermentas, Burlington, ON) according to the manufacturer's instructions. For each sample, a negative reaction was included, by omission of M-MuLV Reverse Transcriptase enzyme.

Comparative real-time PCR

Comparative real-time PCR using the $\Delta\Delta CT$ method was performed with a Lightcycler SW480 and SYBR Green I Master mix (Roche, Basal, Switzerland). The method comprised 45 cycles of denaturation at 95°C for 10 seconds, annealing at 58°C for 10 seconds and elongation at 72°C for 20 seconds. Each biological sample was run in triplicates with porcine specific primers designed on the basis on alignments with human genes, or with primers used in previous studies (Table 1). H₂O and murine MS5 stromal cell cDNA served as negative controls and pooled brain cDNA from Day 42 porcine fetuses served as endogenous control tissue. Three different reference genes were tested on all the samples; glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) (Kuijk *et al.* 2007), Tata box binding protein 1 (*TBP1*) (Nygard *et al.* 2007) and phosphoglycerate kinase 1 (*PGK1*) (Boda *et al.* 2009). GeNorm (Vandesompele *et al.* 2002) and NormFinder (Andersen *et al.* 2004) were subsequently used to determine the most optimal reference gene.

Statistical analysis

Samples were normalized using the $\Delta\Delta Ct$ method. The ΔCT value was calculated by normalizing the CT value of the target gene with the CT value of the house-keeping gene. The $\Delta\Delta CT$ value was calculated by normalizing the ΔCT value to the reference tissue. Finally, the fold change in gene expression was determined by using the equation $2^{-\Delta\Delta CT}$. In the differentiation experiment, calculation of standard deviations as well as statistical analysis was performed on the $2^{-\Delta\Delta CT}$ value using

one way ANOVA to analyse the difference between treated and non-treated samples with a significance level of $p \leq 0.05$.

Fixation of cells and tissues

pNPCs and differentiated cells were cultured on Matrigel-coated (BD Biosciences) glass coverslips (ThermoFisher Scientific), fixed for 20 min in 4% PFA and stored in 1% PFA at 4°C. As a positive control, the head from a Day 42 fetus was fixed overnight in 4% paraformaldehyde (PFA), frozen in Tissue-tek (Sakura Finetek, Torrance, CA), cut into 5 µm sections using a cryostat (Leica microsystems, Vetzlar, Germany) and stored at -80°C. As a negative control tissue, MS5 cells were cultured as described above on Matrigel coated (BD Biosciences) glass coverslips (ThermoFisher Scientific), fixed for 20 min in 4% PFA and stored in 1% PFA at 4°C.

Immunocytochemistry

Immunocytochemistry comprised 30 min permeabilization in 0.1% Triton-X, 1 hour blocking in 5% Donkey serum (Sigma-aldrich) and incubation over night with primary antibodies (Table 2) in 0.25% BSA, 0.1% Triton X (Sigma-aldrich) in PBS. The following day, cells were washed 3 times in PBS, incubated 1 hour with fluorescent-conjugated secondary antibodies (Alexa Fluor; 1:400) in 0.25% BSA, 0.1% Triton-X in PBS, washed 3 times in PBS, incubated in 0.1 µl/ml Bisbenzimidazole Hoechst (Sigma-aldrich) and mounted in fluorescence mounting medium (Dako, Glostrup, Denmark). The OCs were used to verify specificity of pluripotency markers and the brain of a Day 42 porcine fetus, prepared as described above, was used to verify specificity of neuronal antibodies. Murine MS5 cells were included as a negative control. All specimens were examined using a Leica DMRB fluorescent microscope and Leica Application Suite version 2.81 (Leica Microsystems). For quantification of positive antibody labelling of undifferentiated and differentiated cells, a minimum of 200 Hoechst-stained cells from eight different, equally distributed locations on a glass coverslip were counted and compared to the number of cells with positive staining using the freeware quantification program, ImageJ (Collins 2007).

Results

Derivation of pNPCs from epiblast cells

A total of 47 epiblasts were isolated from Day 9 embryos (Figure 2A, B) and cultured on MEF cells and on Day 4, 23 OCs (49%) had formed. Some of the OCs grew as a central dense core, resembling the embryonic epiblast, surrounded by a monolayer of presumptive hypoblast cells, whereas others grew in a monolayer of ESC-like cells (Figure 2C). Both outgrowth types presented typical ESC-like cells with a large nuclear-cytoplasmic ratio containing one or two prominent nucleoli. Following passage of ESC-like cells onto MS5 stromal feeder cells, the cells quickly attached and formed large colonies of cells with ESC-like morphology. Around Day 12-17 round rosettes with a central lumen started to form in the colonies (Figure 2D). When these rosettes were isolated and cultured in Matrigel-coated dishes in N2 medium containing bFGF and EGF, a population of cells rapidly grew out (Figure 2E) presenting a bipolar morphology with large nuclei containing two or more prominent nucleoli (Figure 2F). After the first passage, the cells attained a uniform morphology and neurospheres formed spontaneously during the first three passages (Figure 2G), and when cultured in non-coated dishes (Figure 2H). At present, the cells have been cultured for more than 2 months (16 passages) without losing their proliferation capacity or the ability to generate neurospheres.

Proliferation of pNPCs

Cell doublings were monitored over time by counting individual cells at each passage. Initially, the proliferation rate of pNPCs was exceptionally high with around 10 doublings each passage, however, at later passages this decreased to around 2.5 doublings for each passage, which remained more or less stable (Figure 3A). The proliferation marker KI67, which was examined by immunocytochemistry in pNPCs at passage 3, was expressed 55.8% of the cells (Figure 3B).

Comparative Real-time analysis of pNPCs

To determine the nature of pNPCs, the cells were analysed at passage 1, 3 and 15 by comparative real-time PCR. Reactions were performed with porcine specific primers of NPCs and mature neurons, based on previous characterization of hNPCs (Table 1). All reactions yielded a single transcript and amplicons were confirmed by sequencing. Reference genes tested on all the samples de-

tected consistent levels of transcripts, indicating integrity of the cDNA samples. *GAPDH*, which was determined to be the most optimal reference gene, was subsequently used for normalization.

Expression of the transcription factor *SOX2* was three fold higher in the primary rosette cultures compared to fetal brain (control tissue) (Figure 4A). At passage three, this level was around 1.5 and at passage 15 it was around 0.75, indicating some down-regulation, despite maintaining a relatively high expression. The intermediate filament *NESTIN* followed more or less the same expression profile as *SOX2* with a decrease from three fold to around 0.5 fold the level of the control tissue during passages (Figure 4B). In contrast, the intermediate filament *VIMENTIN* showed a high expression of transcripts at all the examined stages, which was around 5 fold higher than the control tissue (Figure 4C). The transcription factor, *PAX6* was expressed 0.3 fold the level of the control tissue in the rosette cultures, but at passage 3 and 15 the expression was almost undetectable (Figure 4D). Finally, *BETA-TUBULIN III (TUJI)*, and *NCAM*, both considered markers of immature neurons, showed comparable level of transcripts to the control tissue in the primary rosette cultures, but were only expressed 0.1 - 0.2 times the control tissue in the subsequent passages (Figure 4E-F).

Immunocytochemical analysis of pNPCs

Immunocytochemical analysis with an antibody of OCT4 (considered a markers of pluripotency in human and mice), showed nuclear localized OCT4 labelling in OCs, whereas this marker was completely absent in pNPCs at passage 4 (Data not shown). In contrast, quantitative immunocytochemical analyses with previously published hNPC markers (Table 2), showed nuclear localization of *SOX2* in 99.3% of the pNPCs at the same passage (Figure 5A-C). The pNPCs also showed cytoplasmic localization of *NESTIN* (Figure 5D-F) and *VIMENTIN* (Figure 5G-I) in 96.0% and 99.6% of the examined cells, respectively. Furthermore, nuclear localization of *PAX6* (Figure 5J-L) was observed in 99.1% of the cells, which was surprising as transcripts of *PAX6* were barely detectable by comparative real-time PCR. In contrast, *TUJI* (Figure 5M-O) was localized to the cytoplasm in 0.3% of the cells, and *NCAM* staining was negative (data not shown). To further analyse the pNPCs, stainings with GFAP and SSEA1 (considered markers of human radial glial cells) were carried out, however, in both cases stainings were negative (data not shown). Immunocytochemistry performed at passage 16 confirmed nuclear localization of *KI67* and *SOX2* and cytoplasmic staining of *NESTIN* and *VIMENTIN*, whereas, *PAX6* was negative at this stage and a weak cytoplasmic *TUJI* staining was observed in a slightly higher percentage of the cells (Supplementary Figure 1).

Immunohistochemical analysis of Day 42 fetal brain

Immunohistochemical analysis of a Day 42 porcine fetal brain identified specific areas which showed localization of the NPC markers including the developing nasal conchae, the neural layer of the retina, and the ventricular zone (VZ) of the lateral ventricle (Figure 6A). In the VZ, nuclear localization of KI67, SOX2, and PAX6 and cytoplasmic localization of NESTIN and VIMENTIN was observed (Figure 6B-F), whereas, the surrounding marginal zone displayed cytoplasmic localization of TUJ1 and NCAM (Figure 6G-H). Localization of KI67, SOX2, PAX6, NESTIN, and TUJ1 was also observed in the developing nasal conchae and KI67, SOX2, PAX6, NESTIN, VIMENTIN, TUJ1, and NCAM in the neural layer of the retina (Data not shown).

Differentiation of pNPCs into mature neurons and glia

To test the differentiation potential of the pNPCs (Figure 7A), five different protocols were applied. All the protocols comprised removal of the growth factors bFGF and EGF from the culture media and addition of factors to promote the generation of different populations of mature nerve and glial cells.

In the first protocol, without addition of growth factors, the pNPCs mainly attained a multipolar neuron-like appearance with several axons protruding from a single soma (Figure 7B). In the second protocol including RA, SHH, BDNF and AA, the pNPCs again attained a multipolar neuron-like appearance, but in contrast to the previous protocol, extensive clustering of cells was apparent, with long axons protruding from groups of cell soma (Figure 7C). In the third protocol containing FGF8, SHH, BDNF and AA clustering of cells with a multipolar neuron-like appearance was also observed, although to a much lesser extent, and some cells with larger nuclei were observed between the clusters (Figure 7D). At least some of the cells were still proliferating in this protocol and the culture was passaged at Day 9. In the fourth protocol containing PDGF, larger nuclei were observed, often with wide cytoplasmic protrusions (Figure 7E). Again, continued growth required the cells to be passaged at Day 9. Finally, in the fifth protocol containing RA and LIF, a neuron-like appearance was most predominant with pronounced clustering of cells (Figure 7F).

Comparative real-time PCR analysis of differentiated pNPCs

Comparative real-time PCR was performed at the end point of the differentiation experiment. At this point, *NESTIN* expression had decreased to around half the level of undifferentiated cells in

Protocols 1 and 4, whereas, in the remaining protocols the levels were unaltered (Figure 8A). The expression of the early neuronal marker *TUJI* was 10 and 5 times higher than in undifferentiated cells in protocols 2 and 5, respectively, whereas this marker only showed a slight upregulation in protocols 1, 3 and 4. Only protocol 2, however, showed significant difference from undifferentiated cells (Figure 8B). The same expression profile was identified for markers of more mature neurons such as *NEUROFILAMENT (NF)* and *TYROSINE HYDROXYLASE (TH)*, which were both significantly up-regulated in protocol 2 (Figure 8C-D). *GFAP*, a marker of astrocytes, was around 100 fold higher in protocol 1, 2, 3 and 4 which was significantly different from undifferentiated cells (Figure 8E). In contrast, *GFAP* was not statistically different from undifferentiated cells in protocol 5. Finally, a marker of oligodendrocytes, *MYELIN BASIC PROTEIN (MBP)*, was tested, however, this marker was around the detection limit in fetal brain. Nevertheless, it was significantly upregulated around 12 fold compared to undifferentiated cells in protocol 3, whereas, in the other protocols, *MBP* was only upregulated between 1 and 5 times. (Figure 8F).

Immunocytochemical analysis of differentiated pNPCs

Immunocytochemical analysis and quantification of differentiated cells showed that cytoplasmic localization of NESTIN was still observed in 59.0%, 67.8%, 52.2%, 11.5% and 79.5% of the cells in protocols 1, 2, 3, 4 and 5, respectively, indicating that complete downregulation of this marker did not occur (Figure 9A-C). Cytoplasmic localization of *TUJI* was found in 33.7%, 58.0%, 28.6%, 21.3% and 80.0% of the cells in protocols 1-5, respectively (Figure 9D-F), which corresponded relatively well with the observed expression profile of *TUJI*. In addition, cytoplasmic localization of *TH* was observed in protocol 2, but only in few individual cells (Figure 9G-I). Cytoplasmic localization of *GFAP* was observed in 33.8%, 24.5%, 42.6%, 21.9% and 13.3% of the cells in protocols 1-5, respectively (Figure 9J-L), which also correspond more or less with the expression profile of this marker. Finally, cytoplasmic localization of O4, a marker of type I and II pro-oligodendrocytes but not of O-2A progenitor cells (Dhara *et al.* 2008), was observed in protocols 3 and 4 at 29.6% and 63.0% of the cells, respectively (Figure 9M-O).

Discussion

The current study presents for the first time a pNPC line derived from epiblast cells with the ability to differentiate into both mature neurons and glial cells. The pNPCs have currently been cultured for more than 15 passages without losing their proliferative capacity.

Culture of epiblast cells on murine MS5 stromal feeder cells gave rise to formation of rosettes after 12-17 days in culture. The same has been reported for murine and human ESCs cultured under the same conditions (Barberi *et al.* 2003; Perrier *et al.* 2004), indicating that the factors involved in induction of neural fate are conserved between mammalian species. Lazzari and colleagues have previously reported formation of rosettes after 17 days culture of bovine ICM cells on STO feeder cells and shown that the rosettes represent an in-vitro model of early neural specification and differentiation as they respond to the same stimuli as their in-vivo counterparts (Lazzari *et al.* 2006). Hence, porcine epiblast-derived rosettes could be used to study and predict early neural development in the porcine embryo.

In this study, pNPCs were derived and cultured in the presence of bFGF and EGF. bFGF is a well-known mitogen of neural specification, and EGF is reported to promote self-renewal of NPCs (O'Keefe *et al.* 2009). Interestingly, medium containing the same growth factors was also used to derive neural crest precursor cells from bovine ICM cells, which were able to differentiate into neural as well as smooth muscle and cartilage cells (Lazzari *et al.* 2006). However, in the present study, no morphological signs of differentiation to the mesenchymal cell lineage were observed.

An important feature of stem cells is their ability to grow indefinitely in culture, however, this feature is questionable in the case of adult stem cells, such as NPCs. To shed light on this issue, cell doublings were monitored over time. The pNPCs were capable of more than 60 population doublings without ceasing to proliferate, although a initially high proliferation rate followed by a period of slower, yet stable proliferation was observed. This was best described by a logarithmic growth curve, which has previously been reported for hNPCs (Reubinoff *et al.* 2001). However, with the symmetrical divisions of undifferentiated NPCs, one would ideally expect an exponential growth, which has been reported in other studies (Hong *et al.* 2008; Lazzari *et al.* 2006). KI67 staining of the cells at passage 3 showed that 55.8% of the pNPCs expressed this proliferation marker, which is

comparable to the 50% KI67 positive cells found by Du and colleagues (Du *et al.* 2009). In comparison, hNPCs have been shown to express KI67 in 53.5% of the cells, whereas mNPCs express KI67 in 80% of the cells (Sun *et al.* 2009), indicating that pNPCs have similar proliferation rate as hNPCs.

Whereas neural rosettes were positive for most of the markers examined, which indicate a mixed cell population, the pNPCs were only positive for *SOX2*, *NESTIN* and *VIMENTIN* with low expression of *PAX6* as detected by comparative real-time PCR and verified by immunocytochemistry. The prevalence of these markers in most of the cells corroborate their NPC identity and points to a nearly homogeneous population. Furthermore, pluripotent cells did not seem to be present among the pNPCs as staining for the pluripotency marker OCT4 was negative. In comparison, Du and colleagues found that ICM derived pNPCs cells were positive for *NESTIN*, *SOX2* and *VIMENTIN*, whereas *PAX6* was not observed (Du *et al.* 2009), which match our present findings in the pig very well.

Analysis of Day 42 porcine fetal brain using immunohistochemistry showed expression of KI67, *SOX2*, *NESTIN*, *VIMENTIN*, and *PAX6* in the VZ of the lateral ventricle, whereas, *TUJI* and *NCAM* were located in the marginal zone. Hence, the expression profile of the pNPCs derived in this study corresponds well with fetal pNPCs located in the VZ. When Schwartz and colleagues analysed fetal-derived pNPCs with hNPC markers they found expression of *SOX2*, *VIMENTIN* and *NCAM*, whereas *NESTIN* was not detected due to lack of antibodies specificity (Schwartz *et al.* 2005). However, in this study, entire forebrains were used for isolation of NPC, which could potentially include *NCAM* expressing retinal progenitor cells (RPCs). Studies on porcine RPCs have confirmed this expression profile (Klassen *et al.* 2007; Klassen *et al.* 2008).

GFAP is a marker found in several different cell types. It has been shown to be expressed in adult- but not in fetal-derived mNPCs (Imura *et al.* 2003) and in concert with SSEA1, it is frequently used to characterize radial glia in the human brain (Howard *et al.* 2008; Mo *et al.* 2007). The epiblast-derived pNPCs were negative for both GFAP and SSEA1, indicating that these cells share more characteristics with fetal pNPCs derived from the VZ. Du and colleagues found GFAP staining in 13% of pNPCs (Du *et al.* 2009), however, as GFAP is also a marker of type 2 astrocytes (Talbot *et al.* 2002), it is possible that some of these cells had already differentiated into the astrocyte lineage.

For cells to be characterized as true NPCs, ie. multipotent, they must possess the ability to differentiate into both mature neurons and glial cells. To determine the differentiation potential of pNPCs, the cells were subjected to various combinations of growth factors known to promote differentiation of mNPCs and hNPCs into mature neurons and glia.

Generation of mature neurons from pNPCs was most efficient in protocol 2, containing RA, SHH, BDNF and AA. This is not surprising as RA and SHH are known to play key roles in neural patterning in the early embryo (Maden 2007; Patten & Placzek 2000). Interestingly, TH, a marker of dopaminergic neurons, was also found to be present in protocol 2, whereas protocol 3, containing SHH and FGF8, frequently used to generate dopaminergic neurons from hESCs (Perrier *et al.* 2004), TH positive cells were not observed. the different response to these growth factors could be explain by species specific differences. It has previously been shown that a sequential application of FGF8 and SHH promotes formation of dopaminergic midbrain neurons (Hong *et al.* 2008; Yan *et al.* 2005). Thus, the yield of dopaminergic neurons could perhaps be optimized by inclusion of these factors immediately after isolation of rosettes.

Oligodendrocyte progenitor cells were most abundant in protocol 4, containing PDGF as detected by immunocytochemistry. This observation is not in agreement with the expression of *MBP* transcripts, which was significantly upregulated in protocol 3. However, as O4 is a marker of oligodendrocyte precursors rather than mature oligodendrocytes, other factors such as BDNF, GDNF and AA might be required for the final maturation to mature *MBP*-expressing oligodendrocytes. In human, it has previously been reported that low quantities of oligodendrocytes were generated from hESC-derived NPCs (Dhara *et al.* 2008; Reubinoff *et al.* 2001). However, a protocol for generation of large amounts of oligodendrocyte progenitor cells from hESC has recently been published, including 10 days supplementation with RA and SHH followed by 20 days supplementation with bFGF and 2 months maturation in PDGF, insulin-like growth factor 1 and neurotrophin 3, which could be applied to the pNPCs (Hu *et al.* 2009).

Mature astrocytes were efficiently generated in protocols 1 to 4, which is in agreement with the general dogma of spontaneous astrocyte formation (Trounson 2006). Surprisingly, the combination of RA and LIF in protocol 5, which has been shown to yield high number of astrocytes from mNPC (Asano *et al.* 2009) had the opposite effect in this study. It is possible that LIF is responsible for the

inhibitory effect on astrocyte formation as it was the only factor included exclusively in protocol 2. In human, LIF has been shown to stimulate long term culture of hNPCs (Andersen et al. 2009) and it would be interesting to see if the same holds true for pNPCs.

Accumulating evidence suggests that NPCs derived from early-stage embryos possess superior plasticity compared to those derived from older stages. This is illustrated in a study by Chung and colleagues in which ESC-derived mNPCs were able to differentiate into dopaminergic neurons in culture, whereas, fetal brain-derived mNPCs from ventral mesencephalon lacked this ability (Chung *et al.* 2006). Furthermore, transplantation of pNPCs derived from Day 22 and Day 27 embryos into a rat model of Parkinson's disease showed that only the early-stage cells survived the transplantation (Armstrong *et al.* 2003). Harrower and co-workers also found a significant improved survival and integration of in-vitro cultured pNPCs compared to primary porcine grafts in a rat model of Parkinson's disease (Harrower *et al.* 2006). Thus, it seems that in-vitro culture could play an important role in resetting the NPCs to an earlier state, perhaps through erasure of their immunological identity. Since the pNPCs in this study are derived directly from the epiblast and cultured under in-vitro conditions, differentiation, integration and survival in porcine brains might be favoured compared to their in-vivo derived counterparts.

The epiblast-derived pNPCs analysed in this study share many characteristics with hESC derived NPCs such as expression of NPC markers, their capacity for long term proliferation and ability to differentiate into mature neurons and glia. As a result, they may be used in porcine brain transplantation studies as a model of hNPC replacement therapy. Porcine brains share many anatomical and physiological characteristics with humans and pigs have already become the standard experimental model for human brain development (Lind *et al.* 2007). The increasing number of porcine disease models emerging through nuclear transfer (Kragh *et al.* 2009), combined with recent advances in generation of iPSCs may prove to be essential for the study of NPC-based treatment of severe neurodegenerative diseases.

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Figure legends.

Figure 1. Derivation and differentiation pNPCs. The sequential steps for derivation of pNPC as well as the five different protocols used for generation of mature neurons and glial cells.

Figure 2. Morphology of pNPCs. Morphology of pNPCs derived from porcine epiblast cells. (A) Expanded Day 9 in-vivo blastocyst. (B) Isolation of epiblast from the surrounding trophectoderm. Arrowhead points to an isolated epiblast. (C) Epiblast monolayer outgrowth colony grown for 5 days on MEF feeder cells. (D) Neural rosette formed from epiblast cells after 17 days culture on MEF and MS5 feeder cells. Arrow points to a rosette structure. (E) Neural rosette isolated and cultured in Matrigel-coated dishes in medium containing bFGF and EGF. Arrow points to pNPCs outgrowing from the rosette. (F) pNPCs derived from neural rosettes. (G) Neurosphere spontaneously formed during passage 3. (H) Neurospheres formed at passage 15, when cultured on non-coated dishes. Scale bars represent 0,1 mm.

Figure 3. Proliferation of pNPCs. (A) Total cell doublings of pNPCs, estimated by counting of cells at each passage. (B) KI67 staining of pNPCs at passage 3. (C) DAPI staining of pNPCs at passage 3. (D) Merge of B and C. Scale bars represent 0.05 mm.

Figure 4. Expression of NPC markers by pNPCs. Expression of NPC-markers in pNPCs at passage 1 (primary rosettes), passage 3 and passage 15. (A) *SOX2* expression. (B) *NESTIN* expression. (C) *VIMENTIN* expression. (D) *PAX6* expression. (E) *TUJI* expression and (F) *NCAM* expression. The expression was measured by comparative realtime PCR with Day 42 porcine brains as reference tissue. The samples were normalized to the housekeeping gene *GAPDH*.

Figure 5. Immunocytochemistry of pNPCs. Staining of pNPCs with NPC-markers at passage 3. (A) *SOX2*. (D) *NESTIN*. (G) *VIMENTIN*. (J) *PAX6*. (M) *TUJI*. (B, E, H, K, and N) DAPI. (C, F, I, L and O) Merge of primary antibody staining and DAPI. Scale bars represent 0.05 mm.

Figure 6. Immunohistochemistry of porcine fetal brain. Immunohistochemical staining of a porcine brain from a Day 42 fetus. (A) Morphology of the telencephalon, Scale bar represent 0.1 mm. Insert shows a close up of the lateral ventricle with arrows pointing to the ventricular zone (VZ) and the marignal zone (MZ), respectively. Scale bars represent 0.2 mm. (B-H) Antibody stainings of the

VZ and MZ of the lateral ventricle. (B) KI67. (C) SOX2. (D) NESTIN. (E) VIMENTIN. (F) PAX6. (G) TUJI. (H) NCAM. Scale bars represent 0.1 mm.

Figure 7. Morphology of differentiated pNPCs. Morphology after three weeks culture of pNPCs in different growth factors combinations. (A) Undifferentiated pNPCs at passage 3 cultured in the presence of bFGF and EGF. (B) No growth factors. (C) RA and SHH. (D) FGF8 and SHH. (E) PDGF and (F) RA and LIF. Scale bars represent 0.1 mm.

Figure 8. Expression of markers in differentiated pNPCs. Expression of neural and glial cell markers in pNPCs submitted to five different differentiation protocols (protocol 1-5). (A) *NESTIN*, (B) *TUJI*. (C) *NF*. (D) *TH*. (E) *GFAP* and (F) *MBP*. The expression was measured by comparative realtime PCR relative to the expression in undifferentiated pNPCs. Samples were normalized to the housekeeping gene *GAPDH*. The experiments were performed in biological triplicates by differentiating the cells at passage 3. Significant difference from undifferentiated pNPCs is indicated with different significance levels by ***= $p<0.001$, **= $p<0.01$ and *= $p<0.05$. Error bars represent standard error of the mean.

Figure 9. Immunocytochemistry of differentiated pNPCs. Antibody stainings with markers of mature neural and glial cells. Differentiation protocols are shown in bracket. (A) NESTIN (protocol 5), (D) TUJI (protocol 5). (G) TH (protocol 2). (J) GFAP (protocol 3). (M) O4 (protocol 4). (B, E, H, K, and N) DAPI. (C, F, I, L and O) Merge of primary antibody staining and DAPI. Scale bars represent 0.05 mm.

Supplementary Figure 1. Immunocytochemistry of pNPCs. Staining of pNPCs with NPC markers at passage 16. (A) KI67. (D) SOX2. (G) NESTIN. (J) VIMENTIN. (M) PAX6. (P) TUJI. (B, E, H, K, N and Q) DAPI. (C, F, I, L, O and R) Merge of primary antibody staining and DAPI. Scale bars represent 0.05 mm.

Table 1. Primers used for comparative realtime PCR

Primer name	Sequence (5'-3')	Annealing emp.	Product size	Reference
Sox2_Fw	CGCACATGAATGGCTGGAGCAAC	69	182 bp.	TC269881
Sox2_Rv	GGGCGAGCCGTTTCATGTAGGTCTG	71		
Pax6_Fw	GAGTTCTTCGCAACCTGGCTA	60	188 bp.	CU463938.9
Pax6_Rv	TGGTATTCTCTCCCCCTCCTT	59		
Nestin_Fw	GGCAGTGGTTCCAAGGCT	58	161 bp.	XM_001925549.1
Nestin_Rv	GGCTGGCATAGGTGTGTCAA	59		
Vimentin_Fw	GTGATGTCCGCCAGCAGT	57	218 bp.	DQ190948.1
Vimentin_Rv	GCGTTCCAGAGACTCGTT	53		
TujI_Fw	GTGGTGCGGAAGGAGTGTG	59	218 bp.	FP102308.4
TujI_Rv	TGGTGGATGGACAGCGTGG	63		
NCAM_Fw	CGGAGGGAAGCACACGGAG	63	168 bp.	AK237885.1
NCAM_Rv	CGCTTTGCTCTCGTTCTCCTT	61		
TH_Fw	GCACGCCTCCTCGCCCAT	66	181 bp.	CF367294.1
TH_Rv	CTCCACCGTGAACCAGTA	51		
GFAP_Fw	TTGACCTGCGACGGGAGTC	61	225 bp.	Dyce et al. 2004
GFAP_Rv	AGGTGGCGATCTCGATGTCC	62		
MBP_Fw	GAGGCAGAGCTCCTGACTACAAA	61	101 bp.	Zeng et al. 2006
MBP_Rv	GTCCCGTCCTCCCAGCTT	59		
NF_Fw	CAGAGCTGGAGGCACTGAA	57	146 bp.	Zeng et al. 2006
NF_Rv	CATCTCCCACTTGGTGTTCC	57		
GAPDH_Fw	TCGGAGTGAACGGATTTG	54	219 bp.	Kuijk et al. 2007
GAPDH_Rv	CCTGGAAGATGGTGATGG	53		
TBP1_Fw	AACAGTTCAGTAGTTATGAGCCAGA	58	153 bp.	Nygaard et al. 2007
TBP1_Rv	AGATGTTCTCAAACGCTTCG	55		
PGK1_Fw	AGATAACGAACAACCAGAGG	52	126 bp.	Kuijk et al. 2007
PGK1_Rv	TGTCAGGCATAGGGATACC	53		

Table 2. Antibodies used for immunofluorescence

Detection of	Name	Host species	Dilution	Manufacturer
Proliferating cells	Ki67	Mouse IgG	1:100	Dako M7240
ESC	Oct3/4	Goat IgG	1:750	Santa Cruz SC-8628
ESC / NPC	Sox2	Mouse IgG	1:100	RD Systems MAB2018
NPC	Nestin	Rabbit IgG	1:4000	Millipore AB5922
NPC	Pax6	Rabbit IgG	1:4000	Millipore AB2237
NPC	Vimentin	Mouse IgG	1:500	Zymed18-0052
Young neurons	NCAM	Mouse IgG	1:2000	Abcam ab8233
Young neurons	Beta-Tubulin III /TUJI	Mouse IgG	1:4000	Sigma-aldrich T8660
Mature neurons	TH	Rabbit IgG	1:500	Pel-Freez P40101-0
Oligodendrocytes	O4	Mouse IgM	1:250	Millipore MAB345
Astrocytes / Radial glia	GFAP	Rabbit IgG	1:1000	Millipore PAB5804
Radial glia	SSEA1 / LEX	Mouse IgM	1:1000	Biolegend 125602

Figure 1.

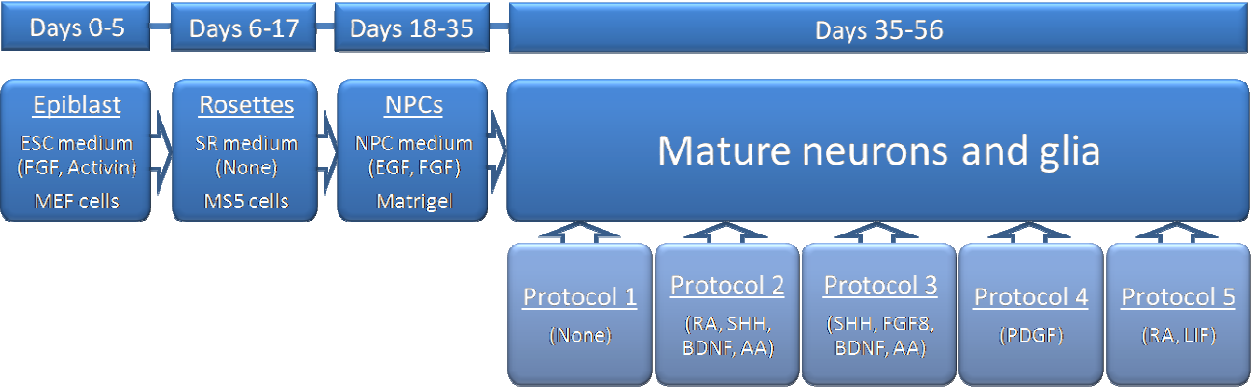


Figure 2.

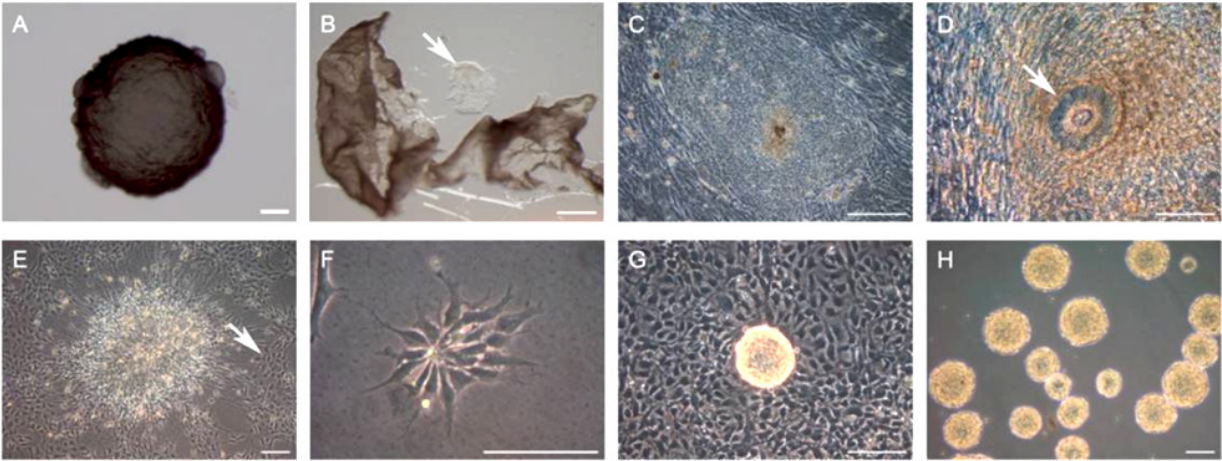


Figure 3.

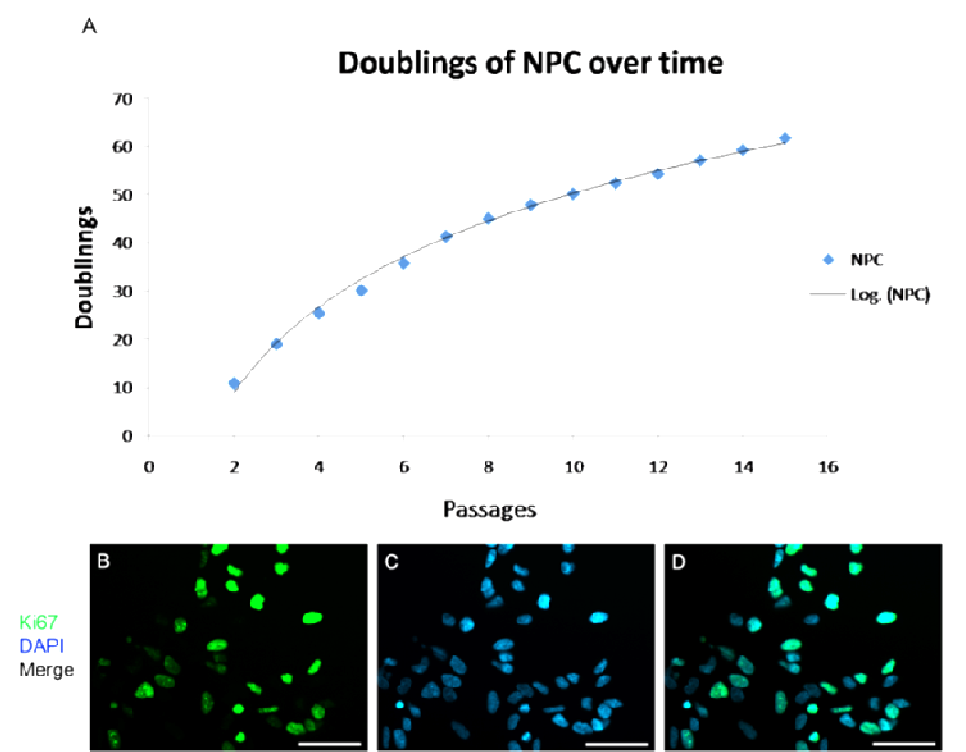


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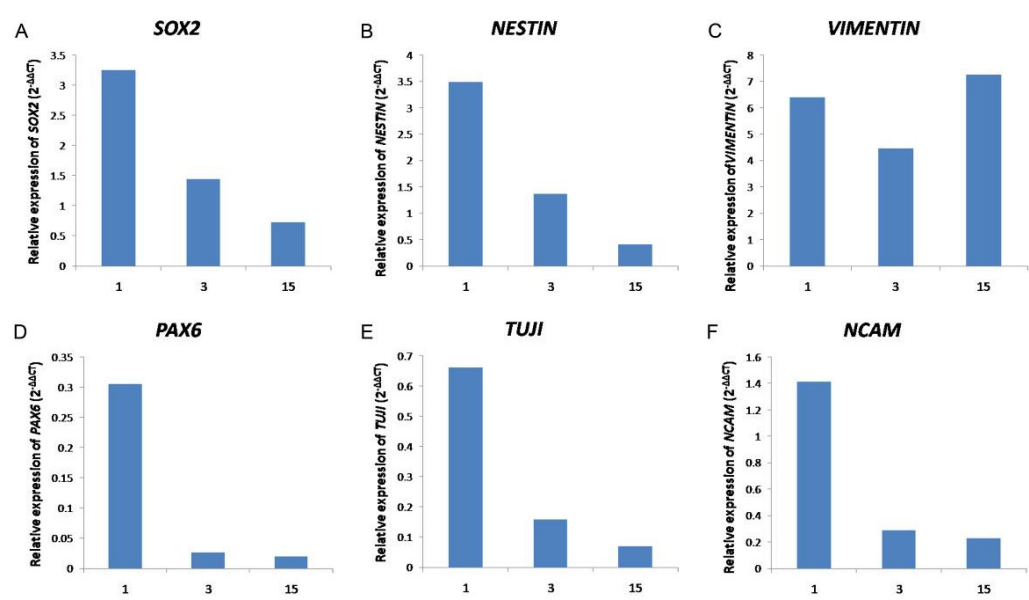


Figure 5.

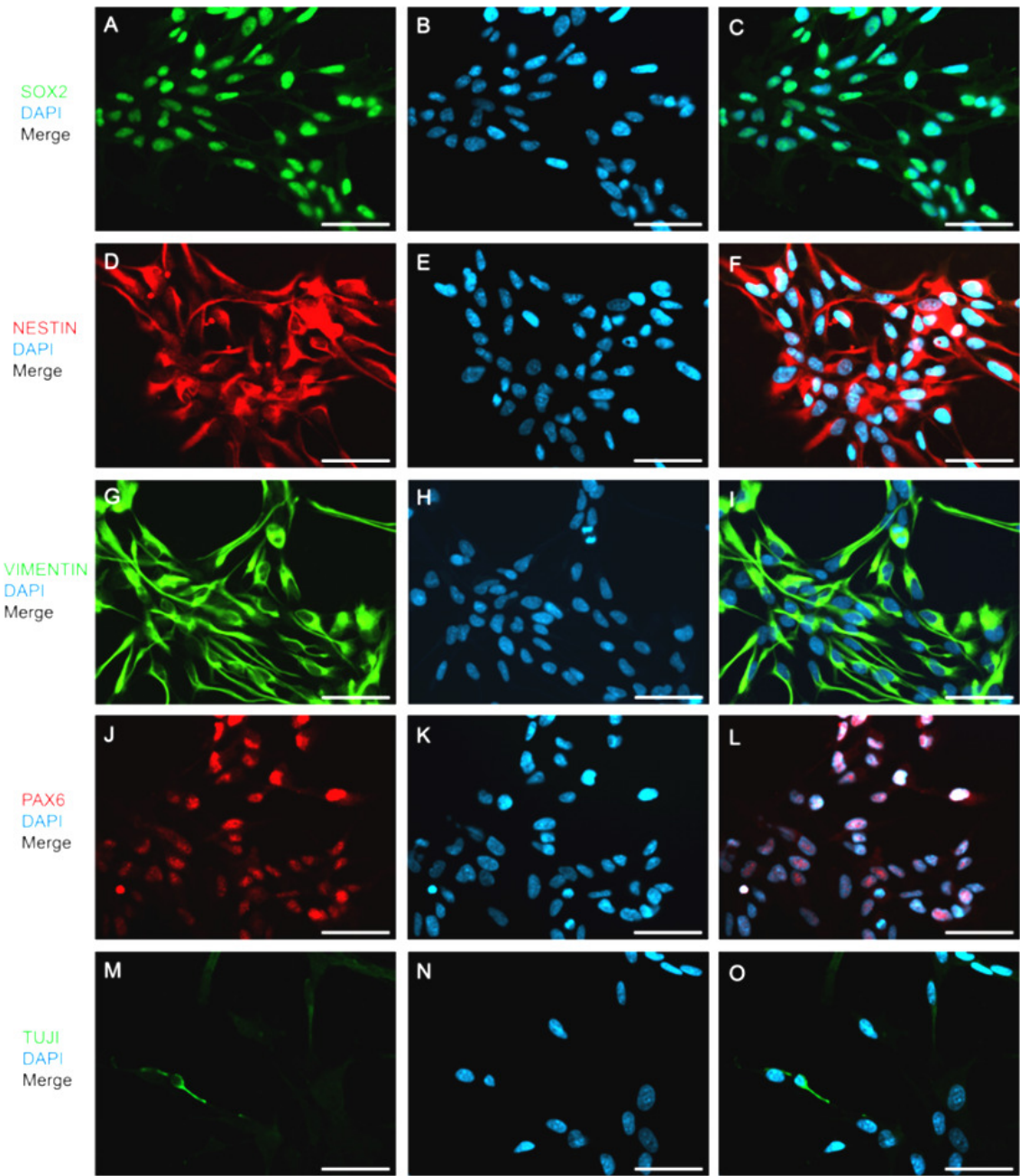


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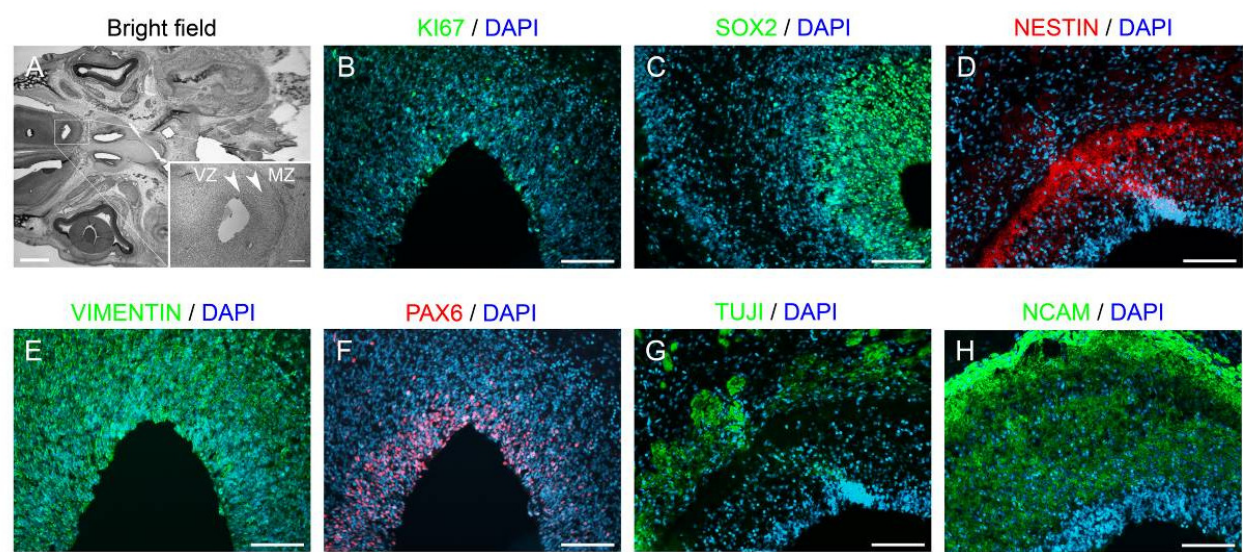


Figure 7.

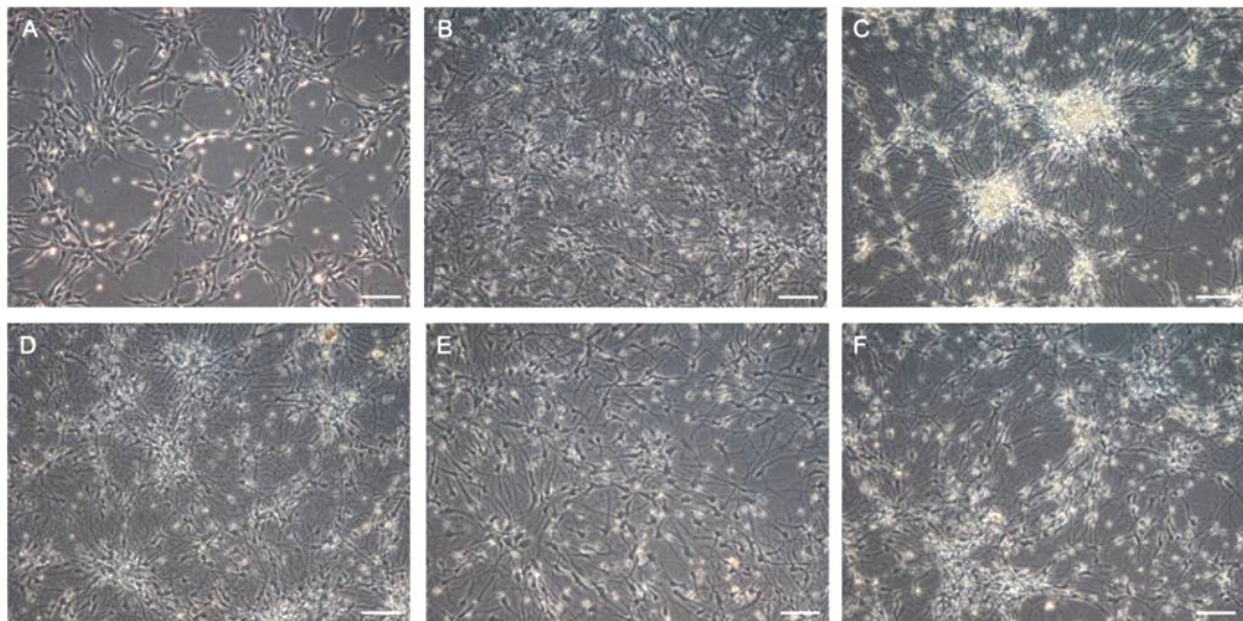


Figure 8.

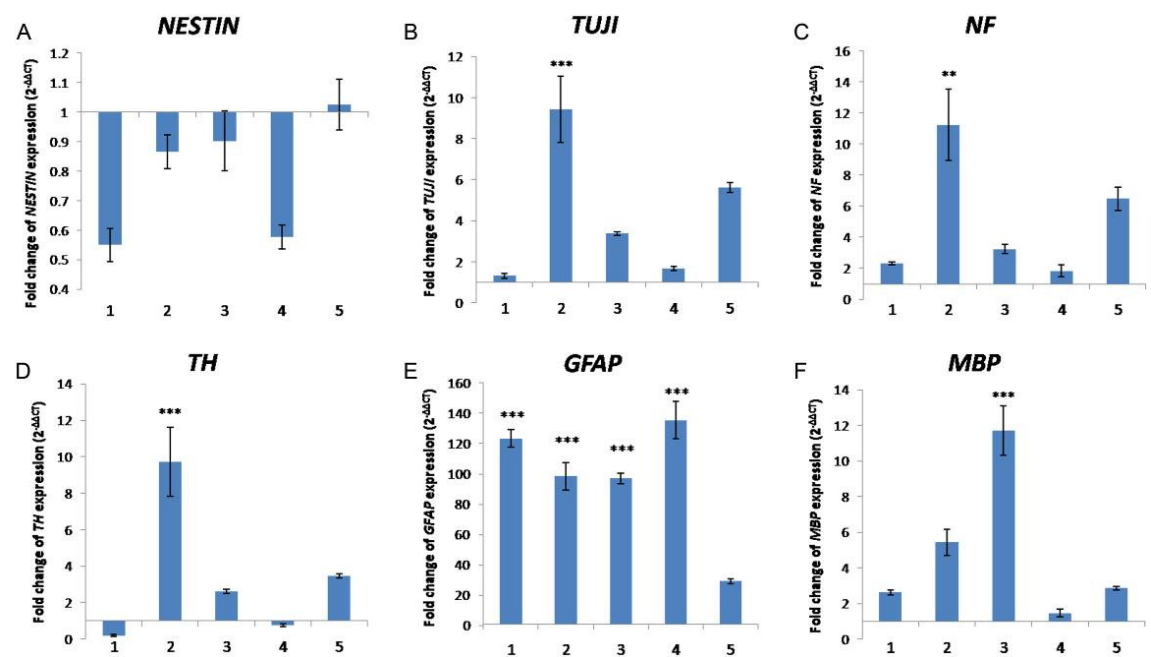
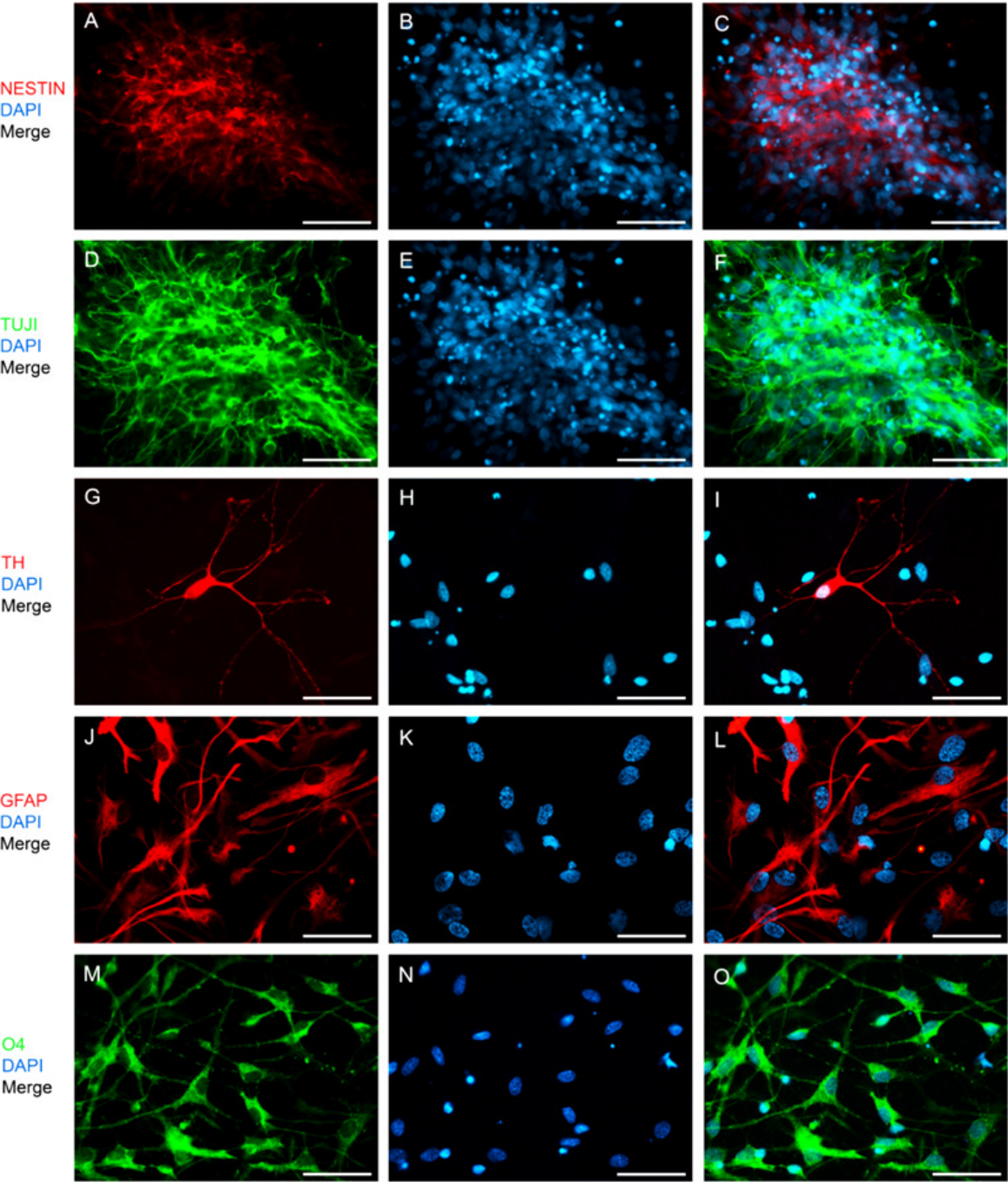
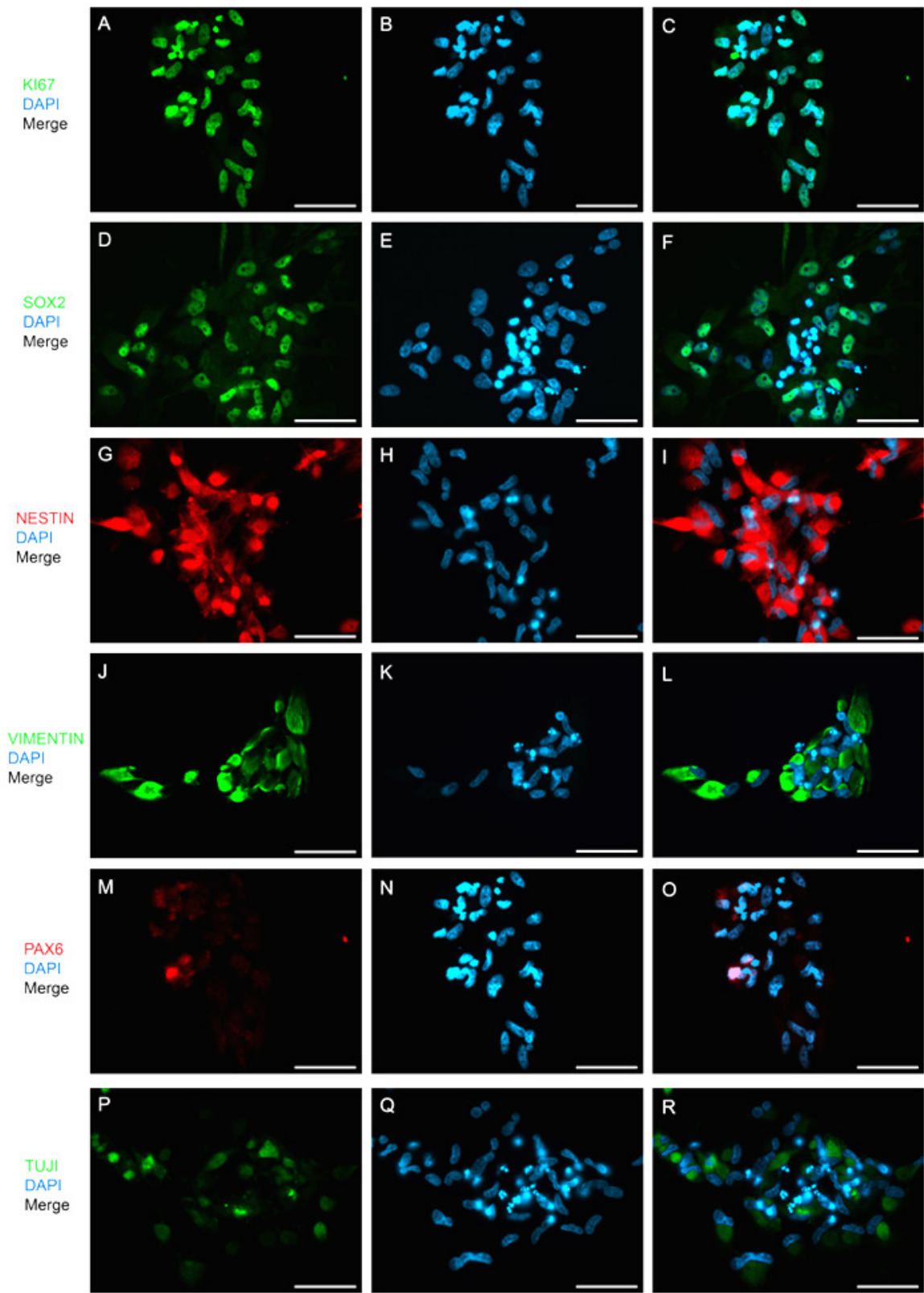


Figure 9.



Supplementary figure 1.



8. Discussion

Developing the pig as a model for cell replacement therapies has become increasingly relevant, considering the need to further investigate stem cell tumorigenesis and disease mechanisms (Oestrup *et al.* 2009). Furthermore, establishment of stable pESC lines would allow for precise genetic engineering either through chimera technology or by improving the efficiency of SCNT technology (Keefer *et al.* 2007). Yet, despite two decades of effort, establishment of pESCs has remained an elusive goal, which may be hindered by some unique features of porcine preimplantation development (Telugu *et al.* 2009).

One of the most important conditions for derivation of ESC cultures is selection of the optimal stage embryo for isolation of the ICM or its derivative, the epiblast (Chen *et al.* 2009). However, the window of opportunity in which to derive ESCs might be different in the pig compared to mouse and human due to a longer preimplantation period (Keefer *et al.* 2007). From a ultrastructural perspective, the ICM of Day 5-6 embryos has been shown to maintain an undifferentiated morphology compared to the epiblast of Day 10-11 embryos, which showed significant specialization containing epithelial features such as tight junctions, interdigitating lateral membranes and microvilli (Janus Jacobsen, unpublished results). However, expression profiling of human pluripotency-related genes has demonstrated that only the epiblast of Day 10-11 embryos showed active transcription of common pluripotency markers, except for *OCT4* which was present in the Day 5-6 embryos as well (Hall *et al.* 2009). Hence, it is of importance to identify the stage of development when the porcine embryo has activated the pluripotent signaling network, but has not yet initiated differentiation, as this is expected to be the best candidate for derivation of ESCs. In this study, both ICM and epiblast cells isolated from Day 5-8 unhatched and hatched blastocysts, respectively, were able to produce ESC-like OCs when subjected to in-vitro culture. Furthermore, isolation of the ICM/epiblast by immunosurgery and manual cleaning appeared to result in the highest attachment rates and ESC-like rates. Yet, despite the initial ability of the ICM and epiblast to convert to in-vitro culture, a general loss of pluripotency was observed after a few initial passages, presumably due to a lack of critical growth factors which are required to prevent differentiation while maintaining proliferation.

Recent evidence points to different signaling factors controlling pluripotency in the ICM and the epiblast, respectively, as EpiSCs derived from the late epiblast stage in the mouse were comparable to hESCs with respect to growth factor requirement and marker expression (Tesar *et al.* 2007). However, since EpiSCs are not capable of germ line transmission (Guo *et al.* 2009), it may be necessary to use the ICM for derivation of germ line capable ESCs in the pig. This is in good agreement with studies showing that live born chimeras with germ line potential could be produced by injection of freshly isolated ICM cells from day 6-7 embryos into blastocysts (Anderson *et al.* 1994; Onishi *et al.* 1994; Nagashima *et al.* 2004). Further studies on the signaling pathways working to maintain pluripotency in different stages of the porcine pre-implantation embryo are required for identification of the factors controlling pluripotency in this species.

The difficulties in establishment of porcine ESC lines may have been exacerbated by the lack of specific markers exclusive to the ICM and its derivative the epiblast (Telugu *et al.* 2009). In the mouse and human, *Oct4* is a candidate regulator in pluripotent and germline cells (Niwa *et al.* 2000). Although OCT4 is localized exclusively in the ICM of the unhatched murine blastocyst (Ovitt & Scholer 1998), this restrictive expression pattern may be the exception rather than the rule, as OCT4 is reported to be localized in both the ICM and trophectoderm of the unhatched blastocyst of other mammals, such as the rhesus monkey, human, pig and cow (van Eijk *et al.* 1999; Kirchhof *et al.* 2000; Mitalipov *et al.* 2003; Cauffman *et al.* 2005). In the human, it was recently reported that the most optimal time for isolation of ESC lines was from Day 6 blastocysts, which correlated with the restriction of OCT4 to the early epiblast (Chen *et al.* 2009). In contrast, in the pig, OCT4 has been reported to be present in both the epiblast and the trophectoderm of the hatched blastocyst (Keefer *et al.* 2007). However, in our study, it was shown that OCT4 was restricted exclusively to the epiblast of porcine Day 9 hatched blastocysts. These contradicting observation could be a result of different OCT4 isoforms, as the antibody used in our study recognize the human OCT4A isoform, whereas the antibody used by Keefer and colleagues recognize the human OCT4B isoform expressed in the trophectoderm (Liedtke *et al.* 2008). Using the former antibody, we have furthermore shown that OCT4 was localized exclusively in cells presenting ESC-like morphology in-vitro, which has previously been corroborated on the mRNA level (Blomberg *et al.* 2008). These findings indicate that the OCT4A isoform may constitute a reliable pluripotency marker in the pig, for identification of the optimal stage embryo for derivation of ESC-lines and for monitoring of pluripotency in culture.

Reporters of *OCT4* and *NANOG* could be extremely important tools for defining the optimal stage embryo for derivation of porcine ESCs, as well as for monitoring of pluripotency in culture. Analysis on the promoter sequences of porcine *OCT4* and *NANOG* have revealed a range of conserved binding sites of transcription factors involved in regulation of pluripotency in the mouse and human, indicating a conserved function in the pig as well. A higher sequence identity was observed between ungulates and primates than between ungulates and rodents, suggesting a closer relationship with humans with respect to the regulation of these pluripotency markers. Furthermore, a restrictive expression pattern of *NANOG* to the ICM of Day 7 blastocyst was observed using a green fluorescent *NANOG* reporter, which is indicative of an important function of this transcription factor in maintaining pluripotency as well. Like *OCT4*, it has previously been argued that *NANOG* is not a reliable pluripotency marker in the pig, due to the detection in several mature tissues using RT-PCR (Carlin *et al.* 2006; Blomberg *et al.* 2008; Kuijk *et al.* 2008). In contrast to the mouse, human and cow (Hart *et al.* 2004; Degrelle *et al.* 2005), isoforms of *NANOG* have not been identified in the pig. However, the detection of a partial *NANOG* sequence (AJ877915.1) (Brevini *et al.* 2007b) in porcine pre-implantation embryos and in ESC-like cells which shows 81% homology with the coding sequence of *NANOG* (DQ447201.1) (Carlin *et al.* 2006) could originate from differential splicing. Until the existence of *OCT4* and *NANOG* isoforms has been meticulously analysed, the results of pluripotency marker expression in porcine cells should be interpreted with caution and additional pluripotency markers reported to exhibit tissue-specific expression, including *SOX2* (Hall *et al.* 2009), *REX1* (Blomberg *et al.* 2008) and *SSEA1* (Wianny *et al.* 1997) should be included.

Despite the inability of the epiblast to maintain pluripotency during prolonged in-vitro culture, spontaneous differentiation toward several different lineages including fibroblasts, endoderm-like cells, neuron-like cells, pigmented cells, contracting muscle cells and epithelial-like cells have been reported (Talbot *et al.* 1993; Talbot *et al.* 2002). In the current study, epiblasts from Day 9 embryos subjected to differentiation on MS5 cells, also developed into structures such as pigmented retinal tissue, beating cardiomyocytes and gut epithelial tissue representing the three germ layers ectoderm, mesoderm and endoderm, respectively (unpublished results). These observations could be considered as a proof that the epiblast derived OCs are pluripotent. Furthermore, developing rosettes gave rise to a pure population of NPCs, capable of long-term self-renewal while maintaining their undif-

ferentiated state and directed differentiation of the NPCs yielded cells of the three lineages, neurons, astrocytes and oligodendrocytes, demonstrating their multipotency. To our knowledge, this is the first time a NPC line has been derived directly from epiblast cells. It was previously shown that rosettes derived from bovine blastocysts respond to the same signaling factors involved in neural specification in-vivo (Lazzari *et al.* 2006). In the present study, factors such as SHH and RA, which are considered potent mitogens of neural specification in-vivo, significantly upregulated neural markers in-vitro, suggesting a conserved response of the NPCs to these signaling factors in-vitro.

9. Conclusion

With human stem cell research rapidly approaching a clinical application, the need for a suitable animal model of stem cell therapy has become increasingly apparent. In the current thesis, the initial conditions for adaptation of the porcine ICM and epiblast cells to in-vitro culture was established. Using bioinformatical analysis, a conserved regulation of the pluripotency markers *OCT4* and *NANOG* between the pig and other ungulates, primates and rodents was shown, indicating a conserved mechanism of these markers in maintaining pluripotency in the pig. *OCT4* was furthermore found to be a reliable marker of pluripotency as it was located exclusively in the epiblast of hatched in-vivo blastocysts and in ESC-like cells in-vitro. Furthermore, *NANOG* displayed a restrictive expression in the ICM of porcine in-vitro produced blastocysts using a green fluorescent *NANOG* reporter. Finally, a epiblast derived NPC line, capable of long term self-renewal and differentiation into neurons, astrocytes and oligodendrocytes, was established. Collectively, these results show that OCs derived from porcine epiblast cells are pluripotent, but lack the ability of self-renewal, whereas epiblast derived NPCs are both multipotent and retain self-renewal capacity. Despite the current lack of stable pESC lines, the pig could constitute an important model of human neural stem cell therapy.

10. Perspectives

There are several possible means to identify the pathways involved in pluripotency in the pig, which is most likely maintained by a combination of transcription factors and epigenetic reprogramming (Talbot & Blomberg 2008). Functional studies of OCT4/SOX2 and OCT4/SOX2/KLF4 binding to the promoter of *OCT4* and *NANOG* in ICM, epiblast and trophectodermal cells may help to shed light on these factors. Furthermore, studies on the methylation and histone modification would be highly relevant. In light of the different reports of expression of core pluripotency markers in the pig, a reporter pig of *OCT4* and *NANOG* would constitute a valuable tool to directly observe transcripion in different tissues instead of relying on indirect methods such as RT-PCR.

Recent evidence points to *Klf4* and *C-myc* as key players in reprogramming to a pluripotent state. In the mouse and human, it has been shown that KLF4 interacts with OCT4/SOX2 to upregulate *Nanog* expression in ESCs and iPSCs (Chan *et al.* 2009; Wei *et al.* 2009) and NANOG has in turn been shown to be responsible for instating ground state pluripotency in undifferentiated cells (Silva *et al.* 2009). Furthermore, in the mouse, *Klf4* overexpression has been shown to be sufficient to revert EpiSCs into germ line competent ESCs (Guo *et al.* 2009). *C-myc* has, in contrast, been shown to be involved in epigenetic reprogramming by promoting DNA replication, thereby relaxing chromatin structure (Niwa 2007). In concert, upregulation of *Klf4* and *C-myc* has enabled the derivation of mESCs from non-permissive mouse strains (Hanna *et al.* 2009). Since the ICM and epiblast cells of the porcine blastocyst are reported to have a low levels of endogenous *C-MYC* and *KLF4* expression (Telugu *et al.* 2009), upregulation of these transcription factors in porcine ICM or epiblast cells may hold the key to establishment of stable pESC lines. This could either be achieved by viral transduction or by use of inhibitors which shield the pluripotent cells from differentiation.

Recently, piPSCs have been generated by use of *OCT4*, *SOX2*, *KLF4* and *C-MYC* (Esteban *et al.* 2009; Ezashi *et al.* 2009; Wu *et al.* 2009). These cells would be ideal for studying signaling pathways controlling pluripotency in the pig. An approach for the establishment of pESCs could be to use co-culture with piPSCs or conditioned medium similarly to the approach used by Martin (1981) in which conditioned medium from a teratocarcinoma cell line was used for establishment of mESCs (Martin 1981). Like in the mouse, germline capable piPSC could potentially be isolated by use of the porcine green fluorescent *NANOG* reporter generated in this study.

Despite the successful derivation of pNPCs from porcine epiblast cells, the methods adapted from differentiation of hESCs into NPCs could be further optimized by inclusion of signaling molecules shown to be involved in neural induction in-vivo. Inclusion of ACTIVIN to the ESC medium, which is known to sustain hESC self-renewal (Beattie *et al.* 2005), but also to stimulate mesoderm and endoderm formation in the early embryo (Schier 2003), could have stimulated the formation of endo- and mesodermal structures as well. Instead, it would be interesting to include NOGGIN, as this growth factor has been shown to be involved in the early induction of neuroectoderm in-vivo (Smith & Harland 1992) and to stimulate neural differentiation of hESCs in-vitro (Sonntag *et al.* 2007). Recently, it was shown that differentiation of hESC to NPCs could be obtained in a defined manner by induction with the factors NOGGIN and SB431542 (Chambers *et al.* 2009), which obviate the need of xenogenic cells and compounds, thereby facilitating the use of hNPCs in regenerative medicine and disease modeling. A similar approach could be applied to the neural induction of porcine epiblast cells.

Recently, hiPSCs have been generated from a range of patients, including PD and HD patients (Park *et al.* 2008a), which could be used for syngeneic treatment of patients in the future. With the recent reprogramming of porcine fibroblast cells into piPSCs, these cells could provide an excellent source for differentiation into pNPCs. Combined with the emergence of important porcine disease models of PD, HD and AD (Lind *et al.* 2007), this would constitute an excellent model of neural stem cell therapy of human neurodegenerative diseases.

11. References

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12. Paper

12.1. Paper I. Embryonic stem cells in pig and cattle: Derivation, culture and potential applications